Identification of microbes degrading nematicides and the development of a diagnostic assay for nematicide persistence in soils.

Thesis

Identification of Microbes Degrading Nematicides and the Development of a Diagnostic Assay for Nematicide Persistence in Soils

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ABSTRACT

The potential for enhanced degradation of the carbamate nematicides oxamyl and aldicarb was demonstrated in UK agricultural soils by applying three successive applications in laboratory incubation. Enhanced degradation was observed in all of the soils treated with oxamyl except one, soil OX 11. The reason for the comparatively slow rates of degradation in this soil could not be explained by any physical property of the soil. Enhanced rates of degradation were recorded for nine of the fifteen soils treated with aldicarb. The rate of aldicarb degradation did appear to be affected by low pH and high organic matter content. Enhanced degradation of the organophosphorus nematicide fosthiazate was not observed in any of the ten soils investigated. This was the case irrespective of soil type, pH, organic matter or nematicide-treatment history.

Enhanced degradation of oxamyl was also observed in liquid media containing oxamyl as the sole carbon source. Thirty-six oxamyl-degrading bacteria from seven different soils were characterised by partial 16S rRNA sequence analysis. Twenty-one of these strains showed high similarity to *Aminobacter aminovorans*, 14 demonstrated an equal degree of similarity to the species *A. niigataensis* and *Chelatobacter heintzii* and one was classed as a *Mesorhizobium* sp. Using *mcd* gene specific primers to detect *mcd* gene homologous sequences resulted in the visualisation of *mcd* gene sized bands, under gel electrophoresis, for ten of the 28 isolates tested.

An investigation into the suitability of the enrichment culture method for predicting the potential for enhanced degradation of nematicides in agricultural soils gave promising results. The enrichment culture method mirrored the enhanced degradation results of the incubation study in nine of the 19 soils tested, *i.e.*, enhanced degradation occurred in both the incubation and the enrichment study or it was absent from both. The method was, however, found to be less reliable in low pH soils.

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AUTHOR'S DECLARATION

This thesis is the work of, and has been written by, the author. It is an original piece of work, no part of which has been submitted for any other degree or professional qualification.

Rachel K Osborn

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AWC	Available water capacity
bp	Base pairs
DNA	Deoxyribose Nucleic Acid
DT50	Degradation time to 50% of original concentration
ECE	Extra chromosomal element
HPLC	High Pressure Liquid Chromatography
LD_{99}	Lethal dose required to kill 99% of organisms
***mcd* gene**	*N*-methylcarbamate-degrading gene
MSM	Mineral Salts Media
MSMN	Mineral Salts Media containing a nitrogen source
MSM+OX	Mineral Salts Media containing oxamyl
MSM+G	Mineral Salts Media containing glucose
NA	Nutrient Agar
NB	Nutrient Broth
OM	Organic matter
***opd* gene**	Organophosphate-degrading gene
PCN	Potato cyst nematode
PCR	Polymerase chain reaction
ppm	Parts per million
PRD	Plant root diffusates
SDW	Sterile distilled water
SEM	Soil extract media
TTR	Total toxic residues
WHC	Water holding capacity
1,3-D	1,3-dichloropropene

1. LITERATURE REVIEW

1.1 WHY INVESTIGATE NEMATICIDE DEGRADATION?

In their paper entitled Accelerated Degradation: The European Dimension, Suett *et al.* (1996) concluded that accelerated degradation is reducing the performance of a wide range of pesticides in agricultural soils across Europe. Evidence for the involvement of soil microorganisms in the rapid degradation and ultimate reduced performance of a pesticide was first brought to light by Audus in 1949 during investigations into the degradation of the herbicide 2,4-dichlorophenoxyacetic acid. The reduced efficacy of pesticides, including many insecticides-nematicides, as a result of rapid biodegradation has since been reported frequently, *e.g.,* Rahman *et al.*, 1979; Felsot *et al.*, 1982; Suett, 1987; Suett *et al.*, 1993 and Suett, 1996.

The two potato cyst nematode (PCN) species, *Globodera rostochiensis* and *G. pallida*, are major pests of UK potato crops. Granular nematicides, including aldicarb, oxamyl and fosthiazate, are one of the most important tools used to control PCN, with greater than £8 million being spent by UK growers on nematicides in 1999 to treat an area of approximately 28000 ha (Evans and Haydock, 2000). The area treated with nematicides increased to approximately 37000 ha in 2002 demonstrating the continued importance of these chemicals (Garthwaite *et al.*, 2002). With few chemicals available to replace them, failure of granular nematicides to control the nematode pest could, therefore, result in an expensive problem (Suett, 1991).

This review will detail some of the mechanisms behind the control of PCN by nematicides and the processes involved in their chemical and biological degradation as well as discuss enhanced degradation and the microorganisms involved.

1.2 POTATO CYST NEMATODES

1.2.1 Life Cycle of the Potato Cyst Nematode Species Globodera rostochiensis *and* G. pallida

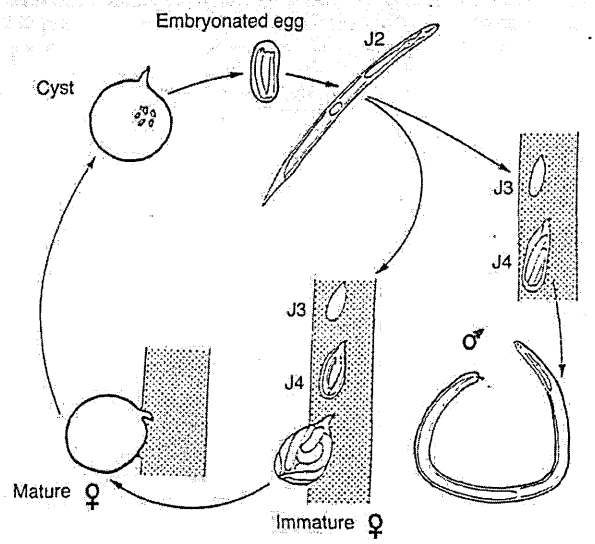


Figure 1: Life cycle of Globodera *spp. (Evans and Stone, 1977)*

1.2.1.1 Hatching

The first stage in the life cycle of the potato cyst nematode (PCN) begins when the second-stage juvenile (J2, Figure 1) is stimulated by chemicals from the roots of the potato plant, known as plant root diffusates (PRD) (Evans and Stone, 1977). Clarke and Perry (1977) proposed that upon exposure to potato plant root diffusates, hatching is initiated by solutes from the egg fluid moving out of the egg, resulting in the increased hydration of the unhatched J2 and a more active metabolism. The unhatched J2 contains only 67% water whilst dormant in the cyst and upon exposure to the potato plant root diffusate the water content of the J2 increases (Ellenby and Perry, 1976). Clarke *et al.* (1978) reported that a 69% water content is necessary for hatched *G. rostochiensis* juveniles to become mobile.

After an increase in the water content, the metabolic activity increases and the nematode is able to cut its way out of the egg. Doncaster and Shepherd (1967), using time-lapse film, were able to document the movements of *G. rostochiensis* J2 within the egg after exposure to potato root diffusate. Juveniles exposed to root diffusate used their stylet to pierce holes through the eggshell, producing a continuous cut to escape through. Once free from the egg the nematode becomes fully hydrated and finally emerges from the cyst (Ellenby, 1974).

1.2.1.2 Invasion of Host Plant and Feeding

Once free in the soil, the nematode must locate and invade the host plant before it utilises all its lipid reserves. It is likely that potato cyst nematodes locate the host using chemical gradients found around the plant's roots (Perry, 1998). The J2 invades the root near the tip and slices its way through cells, until it comes to rest near the root's conductive tissue. Using its stylet, the nematode injects saliva into surrounding cells and withdraws the cell contents. The saliva breaks down cell walls resulting in a large syncytial transfer cell. Nutrients pass from the plant to the syncytial cell where the nematode remains feeding while it develops through the third and fourth stages to the adult (Evans and Stone, 1977). The resulting effect on the potato plant is impaired plant development and reduced tuber yield. With increased invasion by nematodes, plants become stunted and suffer water stress and nutrient shortage and eventually die prematurely causing yield losses (Haydock and Evans, 1998)

Adult males are vermiform and approximately 1 mm long, and once in the soil they can persist for only 10 days in temperate climates (Evans, 1970). At the J3 stage, females begin to develop into a cyst shape. The female dies and the cyst toughens before detaching from the root (Evans and Stone, 1977).

1.2.1.3 Diapause and Quiescence

Potato cyst nematodes employ an obligatory survival strategy to prevent hatch during seasonal periods of predictable, unfavourable environmental conditions; this strategy is termed diapause (Antoniou, 1989). Hominick *et al.* (1985) measured the percentage emergence of juveniles from cysts during alternate months over a year. They found that cysts soaked in PRD under optimal conditions exhibited more prolonged hatching and slower emergence during autumn and early winter than in the following spring and summer. The authors inferred that this may be a survival mechanism to synchronise hatch with the development of the potato plant and that photoperiod effects on the potato plant may send signals to the females and eggs, triggering diapause. Muhammad (1994) conducted a similar study and found almost perfect synchronization of hatch of *G. pallida* with the growing phase of the potato plant. In a later study, Hominick (1986) reared cysts under differing light regimes and found the duration of diapause to be longer for cysts reared in constant darkness compared to the lack of diapause in juveniles in cysts grown on plants in constant light, supplying evidence for photoperiod effects on the duration and initiation of diapause. Temperature is an important environmental signal involved in terminating diapause. Hatch of the cereal root-knot nematode *Meloidogyne naasi* has been shown to be inhibited until exposed to a period of chilling (Antoniou, 1989).

Quiescence is a spontaneous survival strategy triggered as a result of unpredictable, unfavourable conditions and is reversed when conditions improve. For example, unhatched J2s may enter quiescence if host plant root diffusates are absent (Jones *et al.*, 1998). Den Nijs and Lock (1992) demonstrated the dependence of *G. pallida* on PRDs to stimulate hatch, with less than 10% of nematodes hatching in tap or demineralised water. This was a lot less than the hatch of *G.rostochiensis* which demonstrated hatch rates of 60 to 90% in tap and demineralised water, although the ion concentration of the water used may have discriminated against *G. rostochiensis*. It was concluded that plant root diffusate is the

major factor influencing hatch of *G. pallida*, whereas for *G. rostochiensis* other factors may be involved.

1.2.2 Hatch Differences between Globodera rostochiensis *and* Globodera pallida

1.2.2.1 Length of Emergence

There is evidence that *G. pallida* juveniles hatch over a longer period of time than *G. rostochiensis*. Whitehead *et al.* (1984) carried out glasshouse experiments to investigate the invasion of potato cultivar roots by *G. rostochiensis* and *G. pallida*. The authors reported that juveniles of *G. rostochiensis* hatched over a period of 3 weeks, whereas *G. pallida* juveniles hatched over 6 weeks. The longer hatch period of *G. pallida* juveniles is one of the main reasons for its lack of control by granular nematicides, particularly if enhanced degradation occurs (Whitehead *et al.*, 1984; Haydock and Evans, 1998). Whitehead (1992) achieved significantly greater cumulative percentage emergence of *G. rostochiensis* populations than *G. pallida* populations in an *in vitro* study where cysts were exposed to diurnal temperature cycles of 15 °C for 12 h (optimum hatch temperature for *G. pallida*) and 20°C for 12 h (optimum hatch temperature for *G. rostochiensis*) for 6 weeks. By week 6, *G. pallida* populations had a cumulative emergence of only 45.8% (average over 27 populations) whereas a cumulative emergence of 78.6% (average over 12 populations) was observed for *G. rostochiensis*. Similarly, measuring the percentage hatch of *G. pallida* and *G. rostochiensis* juveniles using plant root diffusates from 25 different cultivars, Evans (1983) recorded a mean hatch of 43.4% for *G. rostochiensis* populations and 20.4% for *G. pallida* populations.

1.2.2.2 Temperature

The two species of potato cyst nematode exhibit variation in the optimum temperatures for hatch, with *G. pallida* tending to be adapted to hatch at lower temperatures than *G.*

rostochiensis (Robinson *et al.*, 1987b). This temperature difference between the two species has been shown by many studies, some of which are highlighted in Table 1.

Table 1: Differences in optimum hatching temperatures between Globodera rostochiensis *and* G. pallida.

***G.rostochiensis* Hatching temps**	***G.pallida* Hatching temps**	***Reference***
Min 10°C Max 20°C	Min 8°C Max 16°C	Jones *et al.* (1998)
Dominates in South New Zealand. Mean growing season temperature 15-20°C	Dominates in North New Zealand. Mean growing season temperature 10°C	Foot (1978)
Fastest hatch at 20°C.	Fastest hatch at 10-18°C.	Franco (1979)
3 out of 4 populations showed increase in hatch between 15 and 20°C.	1 out of 4 populations showed hatch increase between 15 and 20°C	Robinson *et al.* (1987b)
60% hatch at 15°C. 20% hatch at 27°C,	40% hatch at 12°C 90% hatch at 15°C 0% hatch at 27°C	Mulder (1988)

The adaptation of *G. pallida* to lower temperatures makes it well suited to the British climate. This also allows *G. pallida* to start hatching sooner than *G. rostochiensis*, thereby allowing it to reach the potato plant roots first, giving it a competitive advantage (Gonzalez and Phillips, 1996). However, Hominick (1979) demonstrated that cultivation practices could lead to the selection for *G. rostochiensis* populations capable of hatch at lower temperatures. A population of nematodes that had been subjected to annual cropping of early potatoes was compared with a population from the same geographic area that had undergone conventional cultivation practices. Hominick found that at 10°C, F1 generation cysts reared in the laboratory from early cropped, field-collected cysts emerged at a faster rate and produced twice as many nematodes as F1 generation cysts from the conventional farm.

1.2.2.3 Lipid Reserves

Robinson *et al.* (1987b) demonstrated differences in the rate of lipid utilisation between the hatched J2s of the two PCN species. They found that at 20°C, *G. rostochiensis* utilised 50% of lipid reserves in 15 days whereas *G. pallida* took 22 days. In a similar study, Robinson *et al.* (1987a) again found slower lipid utilisation in *G. pallida*. The time taken to utilise 50% of lipid reserves during storage in sand saturated with PRD ranged from 10 to 18 days for *G. pallida* J2s and 7 to 11 days for *G. rostochiensis*. Reduced lipid content in the juveniles can be correlated with lowered infectivity and mobility (Robinson *et al.*, 1987a; Storey, 1984). *Globodera pallida* J2s may survive and remain infective in the soil for longer than *G. rostochiensis*, allowing the juveniles to invade potato plant roots after granular nematicides have been degraded to non-effective concentrations.

1.2.2.4 Hatching Factors

As mentioned above, PCN is stimulated to hatch by plant root diffusates, specifically from members of the family *Solanaceae* (Fenwick, 1949). The chemicals within PRDs that stimulate hatch are known as hatching factors (Perry, 1989a) of which only trace amounts are present within the PRD (Devine *et al.*, 1996). Multiple hatching factors have been found to be present in PRD (Devine *et al.*, 1996). Although both *G. rostochiensis* and *G. pallida* are stimulated to hatch by all hatching factors, *G. pallida* tends to respond more to the later produced hatching factors, contributing to the extended period of hatch commonly seen in this species (Bryne, 1996).

Globodera pallida is now the predominant species of potato cyst nematode in England (Whitehead *et al.*, 1991). This was confirmed by Minnis *et al.* (2002) who reported the presence of *G. pallida* in 92% of the potato growing fields surveyed in England and Wales, whereas only 67% contained *G. rostochiensis* only. This predominance is due to the lack of

resistant potato cultivars, its slow decline rate in the absence of the host plant and inadequate control by granular nematicides (Whitehead, 1992). The less reliable control of *G. pallida* by granular nematicides is partly due to the extended period of emergence of *G. pallida* from the cyst, allowing juveniles to invade the roots after granular nematicides have been degraded to non-toxic compounds (Whitehead, 1992). This is illustrated well by Figure 2.

1.3 CONTROL OF PCN

1.3.1 Chemical Control

In the UK, chemical control of PCN is through the application of non-fumigant, granular nematicides and/or fumigant nematicides. The major granular nematicides in use in the UK are aldicarb (Temik, Aventis), oxamyl (Vydate, Du Pont) and fosthiazate (Nemathorin, Syngenta), although fosthiazate was applied to only 7,200 ha in 2002 compared to 20,973 ha treated with aldicarb (Garthwaite *et al.*, 2002). The fumigant nematicide 1,3-dichloropropene (Telone II, Dow Agrosciences) was used on approximately 760 ha in 2002 (Garthwaite *et al.*, 2002).

1.3.1.1 Incorporation of Granular Nematicides

Granular nematicides are applied at planting and protect the plant during its development. The nematicide should persist in the soil at an effective concentration until the plant is large enough to tolerate attack (Smelt *et al.*, 1979). The effectiveness of granular nematicides is dependent on attaining a concentration in the soil that is sufficient to inhibit movement and root invasion by the nematode. This can be achieved by incorporating the chemical into the region of early root development just before planting (Woods and Haydock, 2000a). If the optimum incorporation procedure is not followed the performance of granular, soil-incorporated nematicides may be affected.

Incorporation by rotavators rather than harrows has been shown to obtain better nematode control, mainly due to the better incorporation into the top 15-20 cm rather than the shallower incorporation achieved by the harrow (Smith and Bromilow, 1977). Woods and Haydock (2000b) conducted an investigation into the effect of incorporation depth on nematode control. They found that shallow incorporation (top 10 cm) placed the nematicide above the potato tubers, resulting in little nematode control. Medium incorporation (top 20 cm) gave good control by providing sufficient concentration of nematicide in the soil, whereas deep incorporation reduced the overall concentration of the nematicide at planting making it less effective. Woods *et al.* (1999a) tested a stone separator, bed tiller and a rotavator for granule application and found that the stone separator with the granule applicator positioned at the front of the machine achieved the ideal incorporation depth of 20 cm.

1.3.1.2 Mode of Action of Granular Nematicides

Early studies observing the effects of nematicides on nematodes concluded that granular nematicides act upon the soil phase of the nematode life cycle and do not, as originally thought, control the nematodes after they have entered the root system (Hague and Pain, 1973). The nematicides oxamyl and fenamiphos can also be applied as a foliar application because of systemic action, although applied this way they give little control in the roots. Applying the chemical this way has however, been shown to prevent invasion of roots of cucumber plant seedlings by *Meloidogyne incognita* (Wright *et al.*, 1980). Acetylcholinesterase in nerve tissue is believed to be the target site for organophosphate and carbamate nematicides. Disruption of this enzyme results in impaired larval movement and development, ultimately resulting in starvation (Evans, 1973; Evans and Wright, 1982).

1.3.1.3 Effects on Hatching

Evans and Wright (1982), investigating the combined effect of oxamyl and potato root diffusate on the hatch of *G.rostochiensis*, found oxamyl at a rate of 1.0 μg ml^{-1} (1 part per million, ppm), to partially inhibit hatch; and 4.0 μg ml^{-1} (4 ppm) to completely inhibit hatch. However, the inhibition effects were shown to be completely reversible when the cysts were transferred to PRD alone. Osborne (1973) demonstrated that at rates of 2.5, 5 and 20 mg kg^{-1} (2.5, 5 and 20 ppm) hatch was suppressed in the presence of aldicarb alone, or aldicarb and PRD; however, subsequent hatch after removal to PRD alone was more reduced in cysts exposed to both aldicarb and PRD. The length of exposure to aldicarb also affected hatch, as a greater reduction in hatch was seen with an increase in exposure time. Woods *et al.* (1999b) demonstrated the strong hatch suppression effect of fosthiazate. In pot experiments they showed hatch to be suppressed at concentrations of 1 mg kg^{-1} (1 ppm) and above, and *in vitro* at low concentrations of 0.09 μg ml^{-1} (0.09 ppm). Cyst content data from the pot experiment plotted against soil concentration of fosthiazate did, however, show hatch to increase as nematicide concentrations fell below 0.5 mg kg^{-1} (0.5 ppm).

1.3.1.4 Effects on Mobility and Root Invasion.

Hague and Pain (1970), after applying aldicarb as a drench at a rate of 11.2 kg ha^{-1}, recovered very few second-stage juveniles from the soil or the root system. At a lower dose of 2.24 kg ha^{-1}, hatched juveniles were prevented from entering the roots, giving a satisfactory increase in potato yields. They concluded that the action of the chemical was two-fold, preventing invasion of the roots due to paralysis of the nematode resulting in starvation. Similar effects were seen in their later study (Hague and Pain, 1973) in which a 5 mg kg^{-1} (5 ppm) dose of aldicarb had a very marked effect on root invasion; nematodes were found in the soil but very few were present in the roots. Similarly, Evans and Wright (1982) found oxamyl concentrations of 0.1 and 0.5 μg ml^{-1} (0.1 and 0.5 ppm) to affect the

orientation of nematodes towards the potato roots with movement still impaired after 24 h at the higher dose. This in turn affected root invasion, with very little invasion seen at a dose rate of 0.2 μg ml^{-1} (0.2 ppm).

Fosthiazate has also been shown to affect the mobility of hatched nematodes. In pot experiments, Woods *et al.* (1999b) found concentrations of 1, 2 and 5 mg kg^{-1} (1, 2 and 5 ppm) of fosthiazate to significantly prevent invasion of the potato root system by hatched *G.pallida* juveniles. Also, very few nematodes were recovered from the soil, demonstrating the paralysing effect of fosthiazate. The authors concluded that fosthiazate worked by a combination of hatch inhibition and paralysis of the hatched J2 in the soil. The effect of organophosphorus nematicides on nematode mobility has also been investigated by Karpouzas *et al.* (2005a) using the organophosphorus nematicide cadusafos. Through the use of pot experiments, they established that a low dose of 0.8 μg g^{-1} (0.8 ppm) significantly decreased the number of root-knot nematodes parasitizing tomato plant roots, although nematodes were still counted in roots at the much higher dose of 6.4 μg g^{-1} (6.4 ppm).

It is likely that the two mechanisms, hatch suppression and nematode paralysis, are additive with hatch initially being affected, followed by paralysis or disorientation of the hatched nematode after nematicide concentrations have fallen below that required for hatch suppression (Woods *et al.*, 1999b). Nematodes prevented from reaching their food source will be reliant upon their own lipid reserves, which can become exhausted (Hague and Pain, 1970 and 1973). However, as mentioned previously, *G. pallida* juveniles utilise their lipid reserves at a slower rate than *G. rostochiensis* allowing longer survival in the soil.

1.3.1.5 Reversible Effects of Nematicides

The effects of aldicarb, oxamyl and fosthiazate on hatch and nematode mobility have all been shown to be reversible when the cyst or hatched juvenile are removed from the chemical or when it has been degraded to low concentrations (Nelmes, 1970; Osborne, 1973; Evans and Wright, 1982; Woods *et al.*, 1999b).

Wright *et al.* (1989) exposed *G. rostochiensis* hatched J2s to oxamyl for 35 days to investigate the effect of a long period of oxamyl exposure on lipid utilisation and infectivity. They found that exposure to 1.0 μg ml^{-1} (1 ppm) oxamyl for 35 days paralysed the nematodes from day eight until the end of the study and as a result of reduced movement the oxamyl-treated nematodes had significantly greater lipid reserves than nematodes stored in tap water. When allowed three days to recover in tap water, the root-invasion rate of the oxamyl-treated nematodes was no different to that of the non-oxamyl treated controls. Wright *et al.* (1989) concluded that the lack of decrease in infectivity rates after recovery from the oxamyl treatment was a result of the preservation of lipid reserves. They also suggested that relatively long periods of exposure to nematicides would be required to control *G. rostochiensis* until its lipid reserves are depleted to below a critical level for invasion. As mentioned previously in Section 1.2.2, *G. pallida* utilises its lipid reserves at a slower rate than *G. rostochiensis* posing a greater problem in situations where nematicides are not persisting for long enough. In contrast however, Michaelides *et al.* (2000) reported no measurable recovery of *G. pallida* juveniles after exposure to either oxamyl or fosthiazate for 25 hours.

1.3.1.6 Differential Control of G. rostochiensis *and* G. pallida *by Granular Nematicides.*

As a result of the wide use of *G. rostochiensis* Ro1 resistant potato cultivars in Britain and the suitability of *G. pallida* to the cool British climate, *G. pallida* is now the dominant species in many parts of the country (Turner and Evans, 1998; Minnis *et al.*, 2002).

Globodera pallida is harder to control with granular nematicides than *G. rostochiensis* because of its longer period of hatch, resulting in J2 nematodes hatching after the concentration of nematicide has reached non-toxic levels (Haydock and Evans, 1998), (Figure 2). Whitehead *et al.* (1984) investigated the effects of oxamyl on the control of *G. rostochiensis* and *G. pallida* and found oxamyl to be much less effective in controlling *G. pallida* in field experiments. They concluded that the poor *G. pallida* control could be a result of the extended period of hatch of *G. pallida*. Other studies have however, found equally good control of *G. rostochiensis* and *G. pallida* with granular nematicides, although they were not testing species differences directly (Brown, 1983; Trudgill *et al.*, 1983). In addition, although Whitehead *et al.* (1994) failed to record *G. pallida* control by oxamyl in all field experiments, they did measure increased yields as a result of oxamyl application at some of the sites.

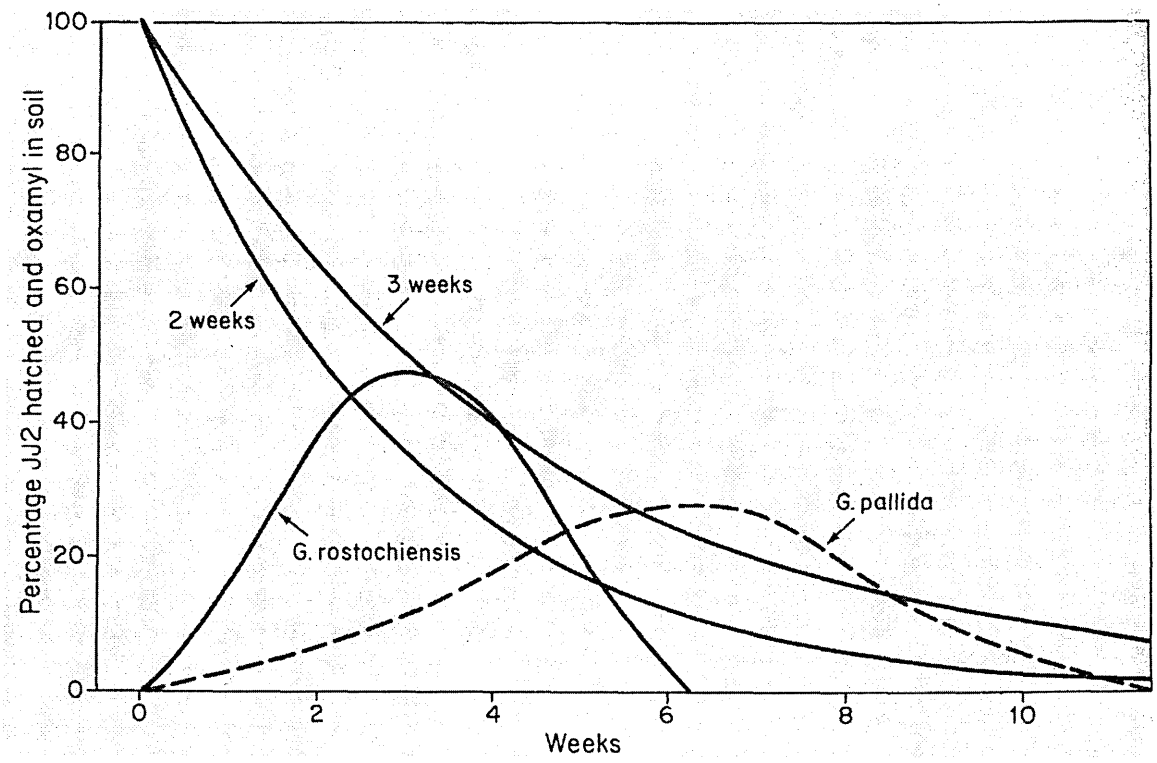


Figure 2: Hatching patterns for G. rostochiensis *and* G. pallida *under a potato crop and decay curves for the granular nematicide oxamyl with 2 or 3 week half-lives (Haydock and Evans, 1998).*

1.3.1.7 Application and Mode of Action of Fumigant Nematicides

The fumigant nematicide 1,3-dichloropropene (1,3-D) has been shown to possess high toxicity to both PCN species (Grove and Haydock, 2000). It can be applied to land at any time during the rotation when a crop is absent. 1,3-Dichloropropene is injected into the soil as a volatile liquid immediately before the applicator seals the soil surface using a roller to prevent the vapour from escaping (Haydock and Evans, 1998; Minnis *et al.*, 2004). Effective fumigation requires specific field conditions. It is important that the soil is not too dry at the time of application as this makes achieving an effective seal difficult, allowing some of the fumigant to escape (Barker *et al.*, 1998). If the soil is water-logged the vapour will not diffuse through the soil and if the soil has a high organic matter content or contains undecomposed crop residues the vapour will be lost through adsorption (Whitehead and Turner, 1998). Low temperatures also affect the efficacy of the fumigant. A large increase in the dosage required to kill 99% of the eggs within a nematode cyst (LD_{99}) is seen at low temperatures of 5°C (McKenry and Thomason, 1974).

1,3-D is effective against nematodes and soil arthropods and, unlike the recently revoked methyl-bromide, is not a general biocide at field rates (Wright, 1981). Fumigant nematicides can be differentiated from granular nematicides by their mode of action, which is to kill the eggs within the cysts, whereas granular nematicides do not kill the nematodes directly (Wright, 1981; Haydock and Evans, 1998). *In-vitro* experiments investigating the effect of 1,3-D in the soil-water phase on PCN hatch demonstrated that a dosage of 25.26 mg l^{-1} per day (50.5 l ha^{-1}) prevented both *G. rostochiensis* and *G. pallida* populations from hatching during the 10 week experiment. The LD_{99} value was calculated as 17.25 mg l^{-1} per day, well below the maximum permitted dose rate within the UK (Grove and Haydock, 2000).

1.3.1.8 Combined use of Granular and Fumigant Nematicides

Granular nematicides only remove nematodes from the population that are surplus to the carrying capacity of the crop (PCN multiplication is density dependent) and so are more effective at reducing yield losses than controlling the PCN population (Evans and Haydock, 2000; Minnis *et al.*, 2004). They are also more effective at preventing an increase in small populations of PCN than they are at reducing large populations (Phillips and Trudgill, 1998). Combined use of a fumigant nematicide with a granular nematicide has been shown to reduce PCN population size and have economic benefits for potato production. Minnis *et al.* (2000) compared the effects of using 1,3-D in combination with aldicarb, oxamyl or fosthiazate on the control of *G. rostochiensis* populations. An autumn application of 1,3-D followed by a spring application of oxamyl was found to give the greatest reduction in eggs g^{-1} soil. Results for the combined use were better than when 1,3-D or oxamyl were used on their own. The combined use of 1,3-D with any of the three granular nematicides had positive effects on the gross margins. The highest gross margin was achieved with the combined use of 1,3-D and oxamyl, with an increase of £2788 ha^{-1} compared with the untreated control. This supports the findings of Barker *et al.* (1998) who also reported the most profitable treatment to be the combined use of 1,3-D and oxamyl when comparing the chemicals separately and when combined. In contrast however, Whitehead and Nichols (1992) found that 1,3-D applied in the autumn at 224 kg ha^{-1} before application of either aldicarb or oxamyl did not provide better nematode control than when aldicarb or oxamyl were applied alone. In a later study, Whitehead *et al.* (1994) found that 1,3-D applied in addition to oxamyl increased tuber yields more than when oxamyl was applied alone but it had little effect on *G. pallida* multiplication. In the MAFF publication, Potato Cyst Nematode: A Management Guide (Parker, 1999), it is advised that because fumigant nematicides are rarely more than 80% effective they should be used in combination with other control measures, such as granular nematicides, to ensure that nematodes surviving fumigation do not multiply.

1.3.2 Integrated Management

Granular nematicides do not always give full control of PCN, particularly *G. pallida* (Section 1.3), and fumigant nematicides are expensive, rarely more than 80% reliable, have to be applied well in advance of the potato crop (because of their phytotoxicity) and require specific soil conditions to work effectively (McKenry and Thomason, 1974; Barker *et al.*, 1998; Whitehead and Turner, 1998; Hockland *et al.*, 2000). To reduce PCN populations, Hockland *et al.* (2000) suggest that granular and fumigant nematicides should be used in combination with other measures, such as preventing the initial infestation of the field, and use of crop rotation and resistant varieties. This approach is known as integrated management. It is also a necessary measure because of concern by environmentalists and consumers over pesticide use, causing supermarkets to put increasingly stringent restrictions on farmers.

1.3.2.1 Crop Rotation

Spontaneous hatch and in-egg mortality of *G. rostochiensis* and *G. pallida* juveniles occur in the absence of the host crop and result in a natural decline in the PCN population. Crop rotation is a strategy used by growers to exploit this and involves growing non-host crops within the rotation for a number of years before growing another potato crop. The length of the rotation depends on the PCN population size and the speed of decline of eggs in the cysts (Whitehead and Turner, 1998). A decline rate of 30% is often used for modelling crop rotation length. Hancock (1988) suggests that a 30% decline would require a rotation length of 7 to 8 years to bring the PCN population down to below the original field infestation level. However, many growers run shorter rotations of 4-6 years resulting in crop losses (Trudgill *et al.*, 1996). Higher decline rates that afford a shorter rotation length have been reported. Devine *et al.* (1999) investigated the decline of *G. rostochiensis* eggs in the absence of the host crop at two sites. They found the decline in viable eggs g^{-1} soil to be 57% one year after potatoes and 40% two years after potatoes at a further site. This

study also reported a temperature effect on spontaneous hatch with a higher rate of hatch occurring during the warmer summer months when soil temperatures were above 10°C.

Nematicides can help to shorten the rotation by decreasing the multiplication rate of PCN when potatoes are grown, allowing more frequent potato production if a longer rotation is not economically acceptable. However, this does depend on the efficacy of the nematicide. With an initially low population of 5 eggs g^{-1} soil and an annual decline rate of 30% over a four-year rotation, the nematicide needs to be 90% effective to prevent the PCN population from increasing between crops (Phillips and Trudgill, 1998). To decrease large populations, a fumigant nematicide followed by the application of a granular nematicide with a resistant potato crop should bring the population under control over a four-year rotation (Phillips and Trudgill, 1998). Ehwaeti *et al.* (2000) predicted, based on an annual decline rate of 30%, that an eight-year rotation would be required to prevent PCN increase when growing Maris Piper (*G. rostochiensis* resistant) on a peaty loam soil combined with the use of a granular nematicide.

1.3.2.2 Resistant Cultivars

Maris Piper, the first available *G. rostochiensis* resistant potato cultivar, was introduced in 1966. Resistant cultivars tolerate nematode attack by preventing the development of juveniles into adult females as a result of insufficient nutrients. Once the nematode becomes established within the root of a resistant cultivar, the feeding site degenerates halting the supply of nutrients to the juvenile (Haydock and Evans, 1998). Some of the *G. rostochiensis* resistant and *G. pallida* partially resistant cultivars used within the UK are listed in Table 2.

Table 2: Some of the more popular potato cultivars within the UK and their resistance status against the two PCN species (Evans and Haydock, 2000)

	Resistance		
Cultivar	G.rostochiensis *(pathotype Ro1)*	G.pallida *(pathotype Pa2/3)*	*Susceptible*
Maris Piper	✓		
Estima			✓
Cara	✓		
Saturna	✓		
Pentland Dell			✓
Nadine	✓	✓	
Hermes			✓
Désirée			✓
Marfona			✓
Lady Rosetta	✓		
Maris Peer			✓
Première	✓		
Maris Bard			✓
Wilja			✓
King Edward			✓
Russett Burbank			✓
Santé	✓	✓	

Minnis *et al.* (2000) reported the very large discrepancy between the percentage of cultivars grown with resistance to *G. rostochiensis* and those with partial resistance to *G. pallida*. Only 6% of 582 cropping records were for *G. pallida* partially resistant cultivars whereas 43% were for *G. rostochiensis* resistant cultivars. The popularity and wide use of *G. rostochiensis* resistant cultivars relative to non-resistant cultivars and those with partial *G. pallida* resistance has played a large part in the rise to dominance of *G. pallida* in British PCN populations (Evans and Haydock, 2000). Out of 484 potato-growing sites sampled around England and Wales, 67% were found to contain pure *G. pallida* populations in 2000 (Minnis *et al.*, 2000). The use of a granular nematicide with *G. pallida* partially resistant cultivars was recommended by Whitehead *et al.* (1994) to control *G. pallida* populations. Similar advice was given by Trudgill *et al.* (2003).

1.4 NEMATICIDE DEGRADATION

Wide variations in the persistence of pesticides in the field have been reported. Ambrose *et al.* (2000) found the disappearance time (DT50) of oxamyl to range from 7 to 28 days in the ten field soils studied. Felsot *et al.* (1982), investigating reasons for the failure of the methylcarbamate insecticide carbofuran to control the corn rootworm in Illinois soils, found percentage recovery of an initial dose to vary from 94% in a silty clay loam to 2% in a loam soil one month after application. The performance of aldicarb and carbofuran against the cabbage root fly has been shown to be negatively affected in a soil treated five times with carbofuran in one year (Suett, 1987).

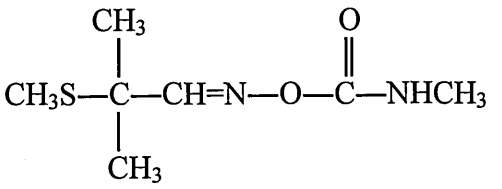
Carbamate and organophosphorus pesticides were introduced to replace the more persistent chlorinated pesticides. They were considered to be more biodegradable because they are converted relatively quickly by hydrolysis to less toxic metabolites (Chapalamadugu and Chaudhry, 1992). Although preventing build-up in the soil, in some cases, such as the examples mentioned above, these chemicals have not been present in the soil at a sufficient concentration to control the target pest. Nematicides need to remain at effective concentrations within the soil to ensure that nematode lipid reserves are depleted, preventing root invasion (Wright *et al.*, 1989) and to provide adequate control of *G. pallida*, which hatches over a longer period of time than *G. rostochiensis* (Haydock and Evans, 1998). When application and incorporation techniques are known to have been optimal, poor performance of nematicides can be attributed to biotic and abiotic factors.

1.4.1 Degradation Pathway of Granular Nematicides.

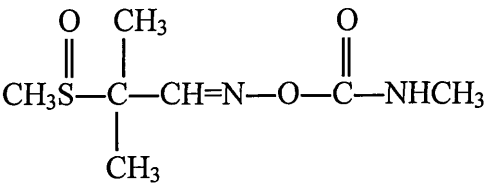
For some chemicals, such as aldicarb and fenamiphos, conversion to more potent compounds is part of their mode of action. Fenamiphos is rapidly oxidised to fenamiphos sulphoxide, which in turn is oxidised more slowly to fenamiphos sulphone (Ou, 1991). Similarly, the methylcarbamate nematicide aldicarb is rapidly converted by oxidation to aldicarb sulphoxide, which in turn is oxidised more slowly to aldicarb sulphone. Aldicarb and its sulphoxide and sulphone remain toxic until they lose the *N*-methylcarbamoyl group via hydrolysis to their respective oximes (Figure 3) (Bromilow *et al.*, 1980; Jones and Norris, 1998). Both fenamiphos sulphoxide and aldicarb sulphoxide have been found to be 10 to 22 times more inhibitory to soil nematodes than their parent compounds (Nordmeyer and Dickson, 1990).

Little measurable oxidation of oxamyl occurs; instead its main path of degradation is via hydrolysis to its non-toxic oximino metabolite (Figure 4) (Bromilow, 1973; Harvey and Han, 1978). The half-life of oxamyl is relatively short. Harvey and Han (1978) reported a half-life of 1 week for oxamyl in field soils. Like oxamyl, the major degradation pathway of carbofuran is thought to occur via hydrolysis of the methylcarbamate linkage, yielding carbofuran phenol and methylamine which can be utilised by microorganisms as a nitrogen and carbon source (Chapalamadugu and Chaudhry, 1992; Feng *et al.*, 1997).

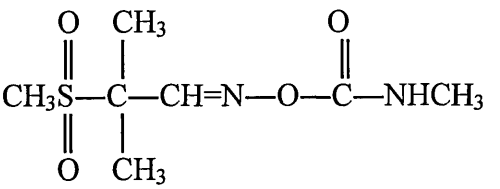
Fosthiazate was introduced to the agricultural market in 1998 and, as such, little research has been conducted into its degradation pathway. However, other organophosphorus pesticides such as fenamiphos (mentioned above), parathion and ethoprophos have been studied. Karpouzas and Walker (2000a) suggested that biodegradation of ethoprophos in soils was via hydrolysis of the P-S bond, resulting in the formation of *O*-ethyl *S*-propyl phosphorodithiolic acid and another metabolite that was subsequently mineralized by



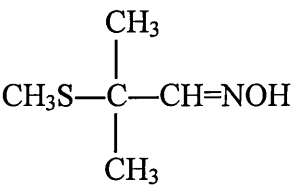
Aldicarb
2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime



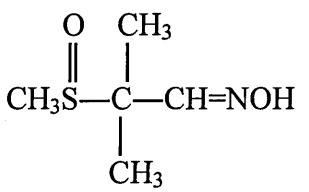
Aldicarb sulphoxide
2-methyl-2-(methylsulphinyl)propionaldehyde *O*-(methylcarbamoyl)oxime



Aldicarb sulphone
2-methyl-2-(methylsulphonyl)propionaldehyde *O*-(methylcarbamoyl)oxime

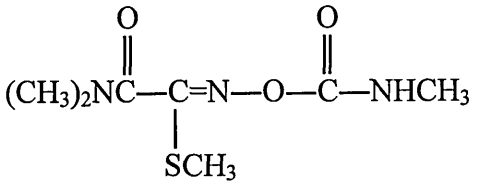


Aldicarb oxime (product of hydrolysis of aldicarb)

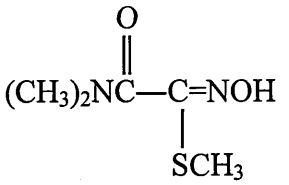


Oxime sulphoxide (product of hydrolysis of aldicarb sulphoxide)

Figure 3: Chemical structure of aldicarb and its oxidation and hydrolysis products (Bromilow et al., 1980).



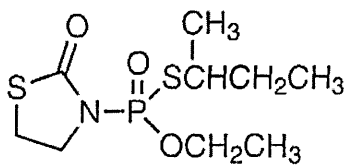
Oxamyl, *N*,*N*-dimethylcarbamoyloxyimino-2-(methylthio)acetamide



Oximino metabolite, methyl *N*-hydroxy-*N*', *N*'-dimethyl-thiooxamimidate

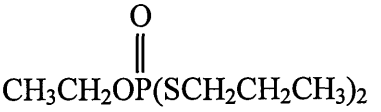
Figure 4: Chemical structure of oxamyl and its hydrolysis product (Bromilow et al., 1980; Harvey and Han, 1978).

adapted soil microorganisms. Similarly, parathion experiences a 120-fold reduction in toxicity upon hydrolysis to its more water-soluble metabolites, diethyl thiophosphoric acid and *p*- nitrophenol (Serdar *et al.*, 1982). Qin *et al.* (2004) suggest that fosthiazate may, like fenamiphos, be oxidised to form sulphoxides and sulphones because of the presence of two sulfur atoms in its chemical structure (Figure 5).

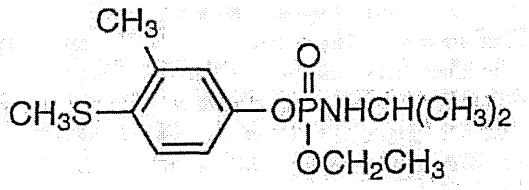


Fosthiazate, (RS)-*S*-sec-butyl *O*-ethyl 2-oxo-1, 3-thiazolidin-3-ylphosphonothioate

Figure 5: Chemical structure of the organophosphate insecticide-nematicide fosthiazate.



Ethoprophos, *O*-ethyl *S*, *S*-dipropyl phosphorodithioate



Fenamiphos, ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate

Figure 5 continued: Chemical structure of the organophosphate insecticides-nematicides ethoprophos and fenamiphos.

1.4.2 Abiotic Degradation of Nematicides

1.4.2.1 Effects of Soil Moisture

Bromilow *et al.* (1980) investigated the degradation of aldicarb and oxamyl under different temperature and moisture conditions and found rates of oxidation of aldicarb and hydrolysis of oxamyl to be slower at a low moisture content of 5% than at 10% or 15%. Similarly, Smelt *et al.* (1979) reported a gradual decrease in the degradation rate of oxamyl with a decrease in the moisture content towards wilting point in a humic loamy sand and a clay loam soil. Gerstl (1984), who also found an effect of soil moisture content on degradation rate of oxamyl, concluded that this relationship may be a result of the effects of the low moisture content on soil microbes. The author postulated that as the soil moisture decreases, the size of the microbial population that can be supported also decreases, causing the lower rate of degradation seen at low moisture contents.

1.4.2.2 Temperature

In an incubation study by Bromilow *et al.* (1980), two sandy loam soils were maintained at temperatures ranging from 5-15°C. When the incubation temperature was dropped from 15 to 10°C or 10 to 5°C the degradation rate of aldicarb decreased markedly, although more of a decrease was seen with the drop from 10 to 5°C. Gerstl (1984) found the effect of temperature on the degradation rate of oxamyl to follow the Arrhenius relationship, with oxamyl being degraded faster at higher temperatures. Similarly, Smelt *et al.* (1978) reported that the conversion rate of aldicarb sulphoxide occurred faster at 25°C than at 15 or 6°C in a clay loam and a greenhouse soil. Half-lives decreased from 77 to 14 days in the clay loam and from 133 to 17 days in the greenhouse soil, with an increase from 6 to 25°C. By summarising the degradation rates of ethoprophos from studies carried out at a range of locations in the United States, Jones and Norris (1998) concluded that temperature seemed to be an important factor affecting ethoprophos degradation. Degradation proceeded faster in the warmer Florida soils than in the cooler Washington soils.

The effects of temperature and moisture on degradation rates appear to be linked. Jukes *et al.* (1996) noted that in a sandy loam at low moisture contents, high temperatures had less of an effect on degradation rates. This was also seen at low temperatures where high moisture contents had less of an effect on degradation.

1.4.2.3 pH

Studies have frequently shown the close relationship between the degradation rates of granular nematicides and soil pH. Harvey and Han (1978) found that the rate of degradation of oxamyl remained stable at pH 4.7 for 11 days, whereas at a higher pH of 9.1, 30% of the oxamyl had been converted within the first 6 hours. Similarly, the half-life of oxamyl was found to range from 13 to 14 days for a clay sand and loamy sand and 34 to 39 days in a peaty sand and humic loam sand (Smelt *et al.*, 1979). They concluded that the

faster degradation in the clay sand and loamy sand was most likely due to the higher pH of these soils. It could, however, be argued that the degradation of oxamyl in the peaty sand and humic loamy sand was also affected by the higher organic matter content of these soils causing greater adsorption reducing bioavailability. A recent study investigating the degradation of fosthiazate in three different U.S. soils found the degradation rate to be slower in the soil with the lowest pH. Half-life values were 26.8 and 17.7 days for soils of pH 7.2 and 6.7 respectively, whereas the half-life for the lower pH soil (pH 5.5) was a much longer 46.8 days (Qin *et al.*, 2004). The degradation rate of ethoprophos has also been shown to be affected by soil pH. In a soil column experiment, the half-life of ethoprophos was reported as 14 and 28 days in a sandy loam (pH 7.2) and a loam soil (pH 7.3). In contrast, in two lower pH soils, a humic sand (pH 4.5) and a peaty sand (pH 4.6), the half-life was 87 days, although the higher organic matter content of these soils may also have had an effect (Smelt *et al.*, 1977).

The hydrolysis of phosphate and carbamate ester bonds is generally base-catalysed and therefore occurs at an enhanced rate in high-pH soils (Wolfe *et al.*, 1990). However, the faster rate of degradation seen at higher pH levels may also be due to the effect of pH on the soil microbial population. Smelt *et al.* (1996) reported highly enhanced degradation rates for aldicarb, oxamyl and ethoprophos in a sandy soil of pH 7.3. Little degradation of these chemicals was, however, observed in a sandy soil of pH 5.6, even though this soil had previously been treated 10 times with all three chemicals. The effect of low pH on the microbial population of that soil may be related to its ability to develop enhanced degradation (Smelt *et al.*, 1996). Read (1987) and Suett *et al.* (1996b) have also reported an effect of pH on microbial adaptation and activity against soil-incorporated pesticides. Low-pH soils may affect the rate at which microorganisms become adapted to degrade pesticides. Suett *et al.* (1996b) observed the effect of pH on lag times. The lag time is the period preceding rapid degradation in which it is suspected that degrading microorganisms

are adapting and increasing in number. They found soils of pH 6.3 and 7.5, previously untreated with carbofuran, to have a shorter lag time than a soil of pH 5.4, in which there was little change in the rate of degradation after 12 weeks. Also of interest is the study by Walker *et al.* (2001) in which they calculated a strong negative relationship between DT50 and soil pH when the spatial variation in degradation rate of isoproturon within a single field was investigated. Results also indicated a significant influence of soil pH on microbial community structure. Investigating this connection between pH and spatial variability further, Bending *et al.* (2003) examined the effect of pH on the metabolism of isoproturon by a *Sphingomonas* sp. previously isolated from the field (Sorensen *et al.*, 2001a). The *Sphingomonas* sp. isolate demonstrated a narrow pH range within which it was able to rapidly degrade the herbicide with optimum activity around pH 7.5, activity dropped above this pH and below pH 7.0.

1.4.2.4 Adsorption

It is generally considered that sorption limits pesticide degradation as a result of reduced bioavailability to soil microbes, potentially causing increased persistence of the chemical, and that the physical and chemical characteristics of soils influence the rate of adsorption and leaching of pesticides (Walker, 1989; Weber *et al.*, 1993). With most pesticides the main factor controlling adsorption in the soil solution is organic matter content, although the hydrophobicity of the chemical will also affect the degree to which it is adsorbed. M'Rabet *et al.* (2002) found that organic matter content accounted for 88% and 98% of the variance in adsorption of fenamiphos and carbofuran respectively when tested in six Moroccan soils, and that due to its greater hydrophobicity fenamiphos showed much higher adsorption than carbofuran. Oxamyl is relatively soluble in water, with a solubility of 280 g l^{-1} (Tomlin, 2004), and as such would be expected to be less affected by adsorption. Gerstl (1984) studied the behaviour of oxamyl in five Israeli soils of neutral to alkaline pH and organic matter contents ranging from 0.11% to 2.03% and found it to be

only weakly adsorbed, resulting in high mobility through the soil. One of the few studies investigating the degradation of fosthiazate reports its adsorption in three soils differing in their physical properties. This study by Qin *et al.* (2004) tested fosthiazate in two sandy loam soils of 0.45 and 0.82% organic matter (OM) and a clay loam of 3.1% OM. They found adsorption of fosthiazate to be negligible in the sandy loam soils and slightly increased in the clay loam. The authors concluded that the slight increase in adsorption in the clay loam may be due to the higher organic matter content of this soil and that the weak adsorption in all three soils may imply that fosthiazate has a high leaching potential.

The organic matter content affects the microbial population, with generally greater microbial biomass occurring in more organic soils (Walker, 1989). However, reduced bioavailability through adsorption has been shown to cause a decline in degradation rates of pesticides. Guo *et al.* (1999) investigated the adsorption and degradation of aldicarb in soil amended with increasing amounts of activated carbon. An increase in adsorption and a decrease in the degradation rate of aldicarb was seen with increasing levels of activated carbon, demonstrating the inhibitory effect of adsorption on degradation. As more aldicarb entered the sorbed phase, degradation slowed with degradation occurring 38 times faster in the liquid phase than in the sorbed phase. It should be noted however that the use of activated carbon might give estimates of degradation rate that deviate from those seen in natural soil. There is also evidence to suggest that variation in the ability to degrade adsorbed chemicals exists between different bacteria. Guerin and Boyd (1993) investigated the bioavailability of sorbed naphthalene to two different bacteria in soil amended with slurry. One of the organisms (*Pseudomonas putida*) was able to continue to mineralise naphthalene at a rapid rate regardless of the organic carbon concentration of the soil/slurry mix, whereas an unidentified bacteria (isolated from petroleum-contaminated soil) demonstrated a decreasing mineralisation rate with increasing slurry concentration and naphthalene sorption. The authors concluded that the bioavailability of sorbed

naphthalene to microbial degraders depends on the organism in question and that the *Pseudomonas putida* strain was able to access and mineralise surface-bound residues resulting in the subsequent desorption of internally bound residues.

Evidence also exists for a time effect on adsorption, with increased sorption and reduced bioavailability occurring with increasing age of pesticide residues. Using a soil contaminated with carbaryl (a weakly sorbed and easily degraded pesticide) 12 years previously, Ahmad *et al.* (2004) investigated the bioavailability of the residues. After inoculation of the soil sample with carbaryl-degrading bacteria, only the water-extractable fraction was degraded with 45% of the carbaryl residues remaining, demonstrating that bioavailability of pesticides can decrease greatly when residues are in long-term contact with the soil. Similarly, Walker *et al.* (2004) investigated the degradation and availability of herbicides over time in two soil samples. Rate of loss through degradation of total extractable residues was slower than the rate of loss of the aqueous extractable residues as measured over 70 days, indicating a decrease in availability over time due to increased sorption. Guerin and Boyd (1993) suggested that the decreased mineralisation rates of naphthalene over a one-year study were a result of residues becoming sorbed more deeply within the soil particle matrix over time.

1.4.2.5 Leaching

Leaching, like adsorption, is a problem because it removes the pesticide from the soil environment making it unavailable to the target pest, but also because if the chemical leaches into surface and ground water it may pose a risk to the water environment and to human health. Adsorption controls the availability of a pesticide in the soil-water phase and as such it also affects its potential for leaching. As mentioned in the adsorption section, a number of studies have found that adsorption increases with increasing residence time of the pesticide in the soil. Walker *et al.* (2004) found that this increase in sorption over time

led to a reduction in the availability of herbicide residues for leaching, indicating that sorption processes are important in controlling pesticide movement through soils. The mobility of aldicarb and its metabolites has been well documented because of its occurrence in drinking water (Jones and Estes, 1995). Fava *et al.* (2001) found aldicarb and its oxidation products to be highly mobile in soil and that although aldicarb was rapidly transformed to aldicarb sulphoxide it was still present in leachate shortly after its application. As the length of time between application and simulated rainfall increased, the concentration of aldicarb in the leachate declined but that of the oxidation products increased. This supports the findings of Leistra *et al.* (1976) who also reported the high mobility of aldicarb and its oxidation products in soil. They also reported the rapid conversion of aldicarb to its metabolites but unlike the previous study they only found it in low concentrations in the soil column effluent. Aldicarb sulphoxide was found to be the most concentrated compound in the leachate, accounting for greater than 40% of the applied dose in three out of the four soils tested. Studies have also been carried out on the mobility of fenamiphos and its metabolites. However, field studies in which simulated rainfall (5 cm) was applied one day after incorporation of the chemical found little downward movement of either fenamiphos or its metabolites (Johnson *et al.*, 1996). This supports results from a previous study (Johnson *et al.*, 1995) in which neither fenamiphos nor its metabolites were detected below the 0-15 cm layer. This study did however have trouble achieving good fenamiphos recovery rates from soils due to the sampling procedure. Davis *et al.* (1994) found fenamiphos and fenamiphos sulphone to be largely unavailable for water extraction from a 1% organic matter loamy sand; fenamiphos sulphoxide was however found to be much more available to the soil water. This may suggest a high adsorption capacity of fenamiphos, a possible reason for the lack of mobility seen in the study by Johnson *et al.* (1996). M'Rabet *et al.* (2002) also noted the greater adsorption of fenamiphos.

1.4.3 Biodegradation: The Cause of Enhanced Degradation

Enhanced degradation is the phenomenon whereby a soil-applied pesticide is rapidly degraded by a population of microorganisms that have adapted as a result of previous exposure to the same or a similar chemical. Enhanced degradation can often result in the reduced efficacy of a pesticide (Racke and Coats, 1988b). It should be noted that the terms 'enhanced' and 'accelerated' degradation are often used to describe the same process. In this thesis the term 'enhanced degradation' will be used because it better implies the increase in the rate of pesticide degradation after repeated application (Felsot and Shelton, 1993).

The enhanced degradation of a number of pesticides, including some carbamate and organophosphorus insecticide-nematicides, as observed in the laboratory, has been widely researched (*e.g.*, Ou *et al.*, 1994; Charney and Fournier, 1994; Karpouzas *et al.*, 1999a; Sorensen and Aamand, 2003; Karpouzas *et al.*, 2004). Suett (1986) incubated soils that had previously received carbofuran as part of an application programme along with a sample from an area of the same field that had no prior treatment and found degradation to consistently proceed faster in previously treated than in previously untreated soils. Suett hypothesised that even though there were differences in the properties between each pair of previously treated and untreated soil, these small differences were unlikely to have been the major cause of the consistent differences in degradation rate. The enhanced rates of degradation were most likely a result of differences in the microbial populations between each pair of soils. Similarly, Smelt *et al.* (1987) reported enhanced degradation of aldicarb, oxamyl and ethoprophos in annually treated plots but not in untreated ones. Sterilisation of the oxamyl and ethoprophos previously treated soils by autoclaving resulted in drastically reduced rates of degradation, supplying evidence for the major contribution of microbes. They concluded that the repeat applications of nematicide to the field plots may have caused microbial adaptation resulting in the accelerated

transformation observed in the incubation studies. A further example of enhanced degradation is that by Karpouzas *et al.* (1999b) in which a field rate dose of ethoprophos was completely degraded in 21 days in a laboratory incubation study using a soil with a 30-year history of ethoprophos application. Ethoprophos persisted for longer than 56 days in the corresponding previously untreated soil. Mixing 'preconditioned' soil from the previously treated field into the previously untreated sample resulted in a significant increase in the degradation of ethoprophos when compared to the unamended previously untreated sample. A lengthy history of application of the specific pesticide in question is not always required to trigger enhanced degradation. Cases of enhanced degradation have been observed in laboratory studies using soils that had received only one previous treatment (Pussemier *et al.*, 1996; Karpouzas *et al.*, 2001).

It does not always follow that repeated application of a pesticide will result in enhanced degradation under favourable soil conditions. In a long-term study conducted by Bromilow *et al.* (1996) the degradation rate of the fungicide benomyl and the insecticide chlorfenvinphos were found to be unaffected by 20 years of annual application to an experimental field plot. The degradation rate of aldicarb was, however, greatly enhanced. Similarly, Singh *et al.* (2003a) reported the degradation rate of chlorpyrifos to be unaffected in UK soils treated with three successive applications. The addition of a small amount of an Australian soil that did rapidly degrade chlorpyrifos resulted in a greater degradation rate in the UK soils, indicating that these soils lacked chlorpyrifos-degrading microorganisms or specific degradation genes amongst their microbial community.

Adding antimicrobial agents to soil previously treated with a pesticide is a good indicator of the involvement of soil microorganisms in enhanced degradation; it also helps to discriminate between the portion of the soil microflora, fungal or bacterial, that is responsible. Racke and Coats (1988b) found degradation of carbofuran to be inhibited

when previously treated soil was autoclaved or an antibacterial antibiotic (chloramphenicol) was added. However, addition of an antifungal antibiotic (cycloheximide) had little effect on degradation, with rates remaining similar to soil that received no antimicrobial treatments, not only indicating the major involvement of microbes but that bacteria play a greater role than fungi. A similar effect of antibiotics was observed with the herbicides napropamide (Walker *et al.*, 1993) and isoproturon (Cox *et al.*, 1996), and the organophosphate nematicide ethoprophos (Karpouzas *et al.*, 1999a). Conversely, Mercia and Alexander (1990) found neither type of antibiotic to completely suppress degradation of carbofuran, suggesting the involvement of both fungi and bacteria. In this experiment however, streptomycin was used to suppress bacterial growth whereas the other studies mentioned above used the antibiotic chloramphenicol, raising the question of whether streptomycin-resistant bacteria could have been present in the soil. Even so, Qing *et al.* (2003) isolated an *Aspergillus niger* strain from agricultural soil with a history of carbaryl application. The fungus was able to utilize carbaryl as the sole carbon source, resulting in its rapid degradation.

1.4.3.1 Isolation of Pesticide-Degrading Microorganisms

A number of studies have successfully isolated individual bacterial strains capable of rapidly degrading certain pesticides from soils demonstrating enhanced degradation (Table 3). Through successive enrichment in selective liquid media containing the target pesticide as the sole carbon or nitrogen source (or both), a range of pesticide-degrading organisms has been isolated.

In some cases, similar species have been isolated from geographically diverse soils. Karpouzas *et al.* (2000a) isolated two similar carbofuran-degrading *Chrysobacterium indologenes* strains from both a UK agricultural soil and a Greek agricultural soil. Parekh *et al.* (1995) isolated similar strains from soils taken from fields at two different sites

Table 3: Pesticide degrading microorganisms

Pesticide Degraded	*Bacteria isolated*	*Reference*
Carbofuran, also degraded carbaryl & aldicarb	*Achromobacter* sp. strain WM111	Karns *et al.* (1986)
Carbofuran	*Pseudomonas stutzeri* *Bacillus pumilus*	Mohapatra and Awasthi, (1997)
Carbofuran, also degraded carbaryl & bendiocarb.	Strain ER2 gram –ve, rod	Topp *et al.* (1993)
Carbofuran	*Sphingomonas* sp. CF06	Feng *et al.* (1997)
Carbofuran, also degraded trimethacarb, isofenphos, cloethocarb, fonofos, ethoprop & phorate	*Streptomycetes pilosus*	Gauger *et al.* (1986)
Carbofuran	Strain MS2d Non-motile, gram –ve rod	Head *et al.* (1992)
Carbofuran, also degraded carbaryl & aldicarb	*Pseudomonas* sp. *Flavobacterium* sp.	Chaudhry and Ali (1988a)
Carbofuran	*Sphingomonas* sp.	Ogram *et al.* (2000)
Carbofuran	*Sphingomonas* sp. strain SB5	Kim *et al.* (2004)
Carbofuran	*Pseudomonas* sp. *Agrobacterium* sp.	Parekh *et al.* (1995)
Carbofuran	*Pseudomonas* sp. *Flavobacterium* sp. *Chrysobacterium indologenes*	Karpouzas *et al.* (2000a)
Carbofuran	*Pseudomonas* sp.	Bano and Musarrat (2004)
Carbaryl, also degraded carbofuran & aldicarb	*Pseudomonas* sp.strain CRL-OK	Mulbry and Eaton (1991)
Carbaryl	*Pseudomonas* sp. strain 50552 *Pseudomonas* sp. strain 50581	Chapalamadugu and Chaudhry (1991)
Carbaryl	*Rhizobium* sp. strain AC100	Hashimoto *et al.* (2002)
Carbaryl	*Blastobacter* sp. strain M501	Hayatsu and Nagata (1993)
Carbaryl	*Arthrobacter* sp.strain RC100	Hayatsu *et al.* (1999)
1,3-Dichloropropene	*Pseudomonas cichorii* 170	Verhagen *et al.* (1995)
Atrazine	*Chelatobacter heintzii* *Aminobacter aminovorans* *Stenotrophomonas maltophilia* *Arthrobacter crystallopoietes*	Rousseaux *et al.* (2001)
Ethoprophos	*Pseudomonas putida* strains ep1 and epII	Karpouzas *et al.* (2000b)
Chlorpyrifos	*Enterobacter* sp. strain B-14	Singh *et al.* (2004)
Cadusafos	*Flavobacterium* sp. *Sphingomonas paucimobilis*	Karpouzas *et al.* (2005b)
Isoproturon	*Sphingomonas* sp. strain SRS2	Sorensen *et al.* (2001a)
Parathion	*Pseudomonas diminuta*	Serdar *et al.* (1982)
Parathion	*Flavobacterium* sp. strain ATCC 27551	Mulbry *et al.* (1986)
Methyl parathion	*Pseudomonas* sp.	Chaudhry *et al.* (1988b)
Fenitrothion, also degraded methyl parathion & parathion	*Burkholderia* sp.strain NF100	Hayatsu *et al.* (2000)

within the UK. However, their method of characterisation only allowed for a tentative identification and grouping of the isolates. Similarly, Rousseaux *et al.* (2001) found that 25 atrazine-degrading bacteria isolated from ten different French soils were represented by

only four different species when their 16S rRNA partial sequences were aligned on the GenBank database. The majority of the isolates demonstrated close similarity to the species *Chelatobacter heintzii*. The authors suggest that the enrichment method used, consisting of successive sub-culturing in complex medium supplemented with the herbicide, might not allow isolation of the full range of atrazine-degrading strains within the soil community.

On the other hand, a wide genetic diversity between bacteria able to degrade the same pesticide has also been reported. In an investigation by Desaint *et al.* (2000), the genetic profiles of 128 carbofuran-degrading bacteria isolated from different French and English soils were compared. They reported that the isolates could be separated into 26 different ribotypes. Thirty degrading strains isolated from soils at HRI in England were spread over only four ribotypes, whereas the 71 Montarden (France) isolates could be separated into 21 of the different groups, demonstrating the range of diversity within individual sites. The procedure for isolation of carbofuran-degrading bacteria used in this study is different to that used in the above-mentioned studies, with both aldicarb and carbofuran being used and at different concentrations. This may have enabled isolation of the diverse range of degrading strains seen.

1.4.3.2 Bacterial Consortia

Some bacterial isolates are unable to fully degrade the pesticide in pure culture but do degrade the chemical when part of a consortium of bacteria. Roberts *et al.* (1993) reported the isolation of 124 linuron-degrading bacteria from soil showing enhanced degradation of the herbicide. However, none of the isolates could degrade linuron in pure culture. The artificial construction of a mixed culture also failed to degrade linuron, demonstrating the complex interactions amongst the bacteria present in the linuron-degrading culture. *Pseudomonas* spp. was found to be an important component of the culture. A consortium

of bacteria capable of utilising the antimicrobial compound triclosan has also been reported (Hay *et al.*, 2001). The consortium consisted of six different bacterial strains one of which was found to be largely responsible for the degradation; however, it was unable to mineralise triclosan unless the consortium was present. Similarly, Sorensen *et al.* (2002) reported that although *Sphingomonas* sp. strain SRS2 was capable of degrading the herbicide isoproturon, the degradation rate was much faster when the bacteria was combined in culture with another strain. The percentage of isoproturon degraded within 14 days increased from 10% to 43% when the two strains were combined. The additional strain, SRS1, demonstrated no activity towards isoproturon, suggesting that this strain aided *Sphingomonas* sp. strain SRS2 by supplying additional nutrients. The mechanisms involved in the degradation of pesticides by bacterial consortia can be divided into two general groups. The first involves the dependence of the bacteria on secondary strains for nutrients, as in the studies mentioned above by Hay *et al.* (2001), Sorensen *et al.* (2002) and also in the study by Mohapatra and Awasthi (1997). In the second mechanism, the various members of the consortium act upon the pesticide at different stages of its transformation, one strain's degradation product providing a substrate for another member of the consortium (Sorensen *et al.*, 2002). An example of this is the investigation by Dejonghe *et al.* (2003) in which a linuron-degrading consortium of five bacteria was isolated. Although one main strain, *Variovoraz* sp. strain WDL1, could utilise the herbicide as a carbon and nitrogen source, the degradation rate was greatly increased when this strain was co-cultured with each of the other four strains. Further analysis revealed that the other strains were responsible for the degradation of intermediate products of the linuron degradation pathway.

1.4.3.3 Enhanced Degradation of Fumigant Nematicides

Fumigant nematicides can, in some cases, limit the enhanced degradation of non-fumigant nematicides by reducing the population size and/or enzymatic activity of the

microorganisms responsible (Section 1.6.1). Also, evidence exists for the beneficial effects on nematode control and crop yields when fumigant and non-fumigant nematicides are used in combination (Section 1.3.1.8). However, fumigant nematicides are themselves subject to enhanced degradation. Enhanced degradation of 1,3-D in a soil with a history of 1,3-D application was reported by Ou *et al.* (1995). Half-life values for cis-1,3-D in surface samples from a soil treated six times in the previous 12 years and its corresponding previously untreated control were 8 and 20 days respectively. Trans-1,3-D was degraded at a similar rate with a half-life of 3 days in the previously treated soil and 17 days in the previously untreated control. Two other soils tested, one previously treated ten times although the last application had been 5 years previously and a second soil that had received one previous treatment, failed to exhibit enhanced degradation. Similarly, Chung *et al.* (1999) also reported enhanced degradation of 1,3-D in a soil treated annually for 3 years, although they found the trans-1,3-D isomer to be degraded more rapidly than the cis-1,3-D isomer. The rate of degradation for both isomers was seen to increase with successive annual applications of 1,3-D. Smelt *et al.* (1989) reported the rapid degradation of cis-1,3-D and trans-1,3-D isomers in soils that had received only one or two previous treatments in the field. The authors also reported the inhibition of enhanced degradation by a high dose of 470 mg kg^{-1} in a soil that had demonstrated enhanced degradation at lower concentrations. The high dose most likely had a toxic effect on the soil microflora. One query with this study however, is the lack of previously untreated samples taken from the same or near-by field sites for use as controls, making it difficult to assess whether enhanced degradation definitely occurred.

1.5 ENHANCED DEGRADATION: BEHIND THE SCENES

1.5.1 Adaptation of Soil Microorganisms.

Kearney and Kellogg (1985) have described the adaptive processes that are occurring in soils as a result of repeat applications of herbicides. A pattern is often seen in which

initially there is a small increase in degradation rate, followed by a lag phase where little measurable degradation occurs. This lag phase, the duration of which varies between chemicals and number of treatments, then gives way to a period of rapid degradation. During the lag phase it is thought that the microbial population is adapting by way of population growth, dissemination of genetic material and enzyme synthesis resulting in the rapid degradation of subsequent applications of the chemical without a lag phase (Figure 6). Torstensson *et al.* (1975) noticed this phenomenon with the herbicide MCPA. Soils that had a one-year or 18-year history of MCPA application had a three-fold faster degradation rate than soil that had no previous MCPA treatment. The lag phase for the previously untreated soil lasted 21.2 days, whereas it lasted only 4.3 and 3.5 days for the 1-year and 18-year applications, respectively. The authors concluded that during the lag phase the microbial population may be adapting and increasing in size. They did not, however, find any differences in the size of the microbial population nine months after an application of MCPA when compared with the control. Nine months may have been too long to be able to gain an accurate representation of the effect of MCPA on microbial population size. Measurements taken immediately after the period of rapid degradation may have given different and more reliable results.

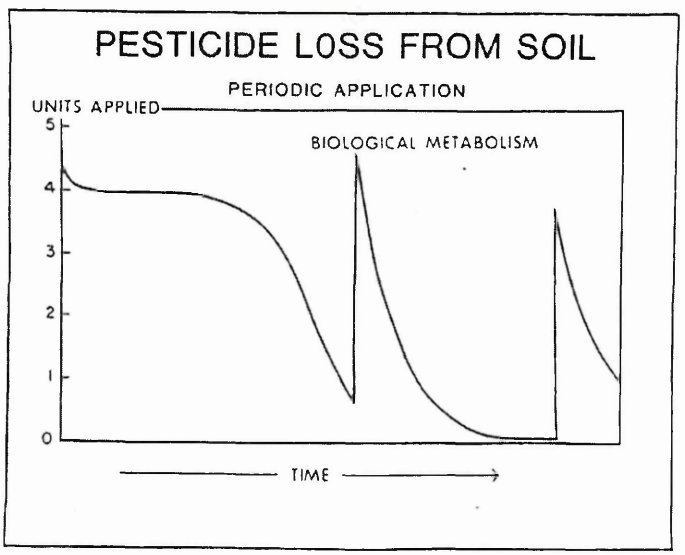


Figure 6: Kinetics of pesticide biodegradation (Kearney and Kellogg, 1985)

It is thought that two strategies are involved in chemical metabolism by microorganisms. One is mineralization, in which the metabolism of the substrate yields energy or nutrients, resulting in an accompanying increase in the biomass of the population of degraders. Subsequently the capacity for degradation would be much greater causing the rapid degradation seen with repeat applications (Felsot and Shelton, 1993). Trabue *et al.* (2001) reported a significant increase in the number of carbofuran hydrolysers after a second application of carbofuran. Similarly, Charnay and Fournier (1994) reported the corresponding increase in carbofuran degradation rate with an increase in the size of the population of bacteria able to utilise carbofuran as a carbon and nitrogen source. Also, larger numbers of degraders were measured in previously treated than in previously untreated soils. Bending *et al.* (2003) also reported an increase in the size of the population of isoproturon degraders in soil samples treated with an application of isoproturon. Prior to the addition of isoproturon the mineralising community averaged 5 x 10^3 degraders g^{-1} soil, at the point of 90% isoproturon degradation the number of degraders had increased to 2.5 x 10^6 g^{-1} soil. In soils that exhibited co-metabolism of isoproturon there was no evidence of population increase with a subsequent isoproturon application. In contrast, Desaint *et al.* (2000) observed faster degradation rates of carbofuran and aldicarb in a soil with a smaller population of carbamate-degrading bacteria than a soil that had received no carbamate applications for 15 years. Mercia and Alexander (1990) reported similar findings. They found that although degradation rates of carbofuran were high there was no significant difference in the number of carbofuran degraders between pesticide treated and untreated samples. Sterilisation of the soil sample reduced the degradation rate, demonstrating that the process was chiefly microbial. The authors suggested that the addition of carbofuran to an already large population of carbofuran degraders would not result in a detectable increase in cell numbers. Similarly, enhanced degradation of the carbamothioate herbicide EPTC with no concurrent increase in the size of the degrading population has been reported (Moorman, 1988). Again, soil sterilisation substantially

reduced the rate of degradation, confirming the role of microorganisms. Moorman suggested that EPTC might not meet all nutritional requirements necessary for growth.

The second strategy involved in pesticide degradation is known as co-metabolism. Co-metabolism yields no energy from the metabolism of the pesticide and as a result no accompanying increase in the size of the degrading population occurs (Felsot and Shelton, 1993). Co-metabolism may result from the close resemblance of a pesticide molecule to that of an enzyme's natural substrate, causing the catalysed transformation of the pesticide (Karns, 1990). Co-metabolism would only cause enhanced degradation if conditions were favourable for microbial activity, such as when a soil has been amended with sewage sludge (Felsot and Shelton, 1993). Robertson and Alexander (1994) conducted a study to investigate growth-linked degradation and co-metabolism. An increase in the rate of degradation of 2,4-D, glyphosate and propham was seen with a second application of these chemicals applied 10 days after the first. This was accompanied by an increase in the size of the degrading population. Mineralization of the herbicide simazine was slow, although an increase in the degradation rate was seen in previously treated soils when compared to previously untreated sample. No corresponding increase in the number of degrading microorganisms was seen and little incorporation of simazine ^{14}C into the microbial biomass was measured, suggesting that siamzine was co-metabolised. The increased rate of degradation of simazine seen after the second application may therefore, have been a result of an increase in activity per unit of biomass rather than an increase in the size of biomass (Robertson and Alexander, 1994).

1.5.2 Adaptation at the Molecular Level

Co-metabolism is unlikely to be responsible for enhanced degradation but may play a part in the normal rate of degradation. Deriving energy from the transformation of the pesticide resulting in an increase in the population of degraders until the population is large enough

to cause a noticeable effect on the rate of degradation is also unlikely to be the only mechanism involved in enhanced degradation. If this was the case, either enhanced degradation would be expected to occur with all soil-incorporated pesticides or a build-up of persistent residues would occur (Karns, 1990). Increases in the size of the population of degraders may be triggered by genetic events taking place within the soil microbial population. If a mutation in the gene coding for the pesticide hydrolase enzyme caused the enzyme to be produced at a higher rate, a population of bacteria that rapidly degraded the pesticide would result. If the mutated gene was located on an extra-chromosomal element (ECE) such as a plasmid, transposon or insertion sequence, it could be disseminated amongst the microbial community resulting in the rapid adaptation of the bacterial population. ECEs are self-replicating and independent of chromosomal DNA and as a result pose an evolutionary advantage by enabling the bacteria to survive when the environment that the mutated gene was well adapted for returns to 'normal', *i.e.,* the pesticide is removed (Kearney and Kellogg, 1985; Karns, 1990). Genes encoded on ECEs can be readily disseminated amongst the bacterial community by three main mechanisms: transformation, bacteria take up DNA directly from the environment; transduction, genes are passed between bacteria by bacteriophage particles; and by conjugation, the transfer of DNA by cell-to-cell contact (Karns, 1990). The ease of exchange of genetic material would allow the rapid adaptation of a soil microbial community for the advantageous task of utilising a new organic compound within what can be a nutritionally poor environment.

Genes encoding for pesticide hydrolase enzymes have, in some cases, been identified and many have been found to be located on plasmids. Karns *et al.* (1986) isolated an *Achromobacter* bacterium, designated *Achromobacter* sp. strain WM111. This bacterium was capable of rapidly utilizing carbofuran as a sole source of nitrogen, degrading 99% of 200 μg ml^{-1} of carbofuran in 42 hours. Further studies went on to identify the gene involved in the hydrolysis of carbofuran and its location (Derbyshire *et al.*, 1987; Tomasek

and Karns, 1989). The DNA fragment encoding the hydrolase enzyme was found to hybridise strongly to a large plasmid from *Achromobacter* WM111, whereas it hybridised only weakly to total DNA from this bacterium, indicating that the *N*-methylcarbamate degradation gene (*mcd* gene) is encoded on a plasmid (Tomasek and Karns, 1989).

The *mcd* gene has been identified in other carbamate-degrading bacteria isolated from various locations. Topp *et al.* (1993) isolated a Gram-negative bacteria, designated ER2, which hydrolysed carbofuran, carbaryl, propoxur and bendiocarb. Two plasmids, pER2a and pER2b, were detected in this bacterium. Of the two, plasmid pER2a was of a similar size to the plasmid from *Achromobacter* sp. strain WM111. A *mcd* gene probe cloned from *Achromobacter* sp. strain WM111 hybridised with plasmid pER2a. The authors concluded that ER2 and WM111 were genotypically distinct organisms but contained similar plasmids with homology to the *mcd* gene. They also suggested that the *mcd* gene could be useful as a probe for studying enhanced degradation of carbofuran in soils.

The usefulness of the *mcd* gene as a probe is confounded by evidence demonstrating that the *mcd* gene is not the only gene involved in carbofuran degradation and is not always present in methylcarbamate-degrading bacteria. Parekh *et al.* (1995) recovered 55 bacterial isolates from English and French soils that hydrolysed carbofuran to carbofuran 7-phenol. Of these, the majority were Gram-negative bacteria, although a high degree of phenotypic and genotypic diversity was demonstrated amongst the 55 isolates. Most carbofuran-degrading isolates contained one or two large plasmids. However, sequences homologous to the *mcd* gene were only detected in 22 of the 55 isolates. The presence of the *mcd* gene also varied from site to site. Eighty-three percent of isolates from one site at Wellesbourne, England hybridised with the *mcd* gene probe, but no isolates from site C at Wellesbourne or any of the isolates from one of the French samples contained homologous sequences. It appears, therefore, that the *mcd* gene probe would only be a useful tool for monitoring

carbofuran degradation if used in soils where the majority of the hydrolysing population contained genes homologous to the *mcd* gene (Parekh *et al.*, 1995). In a later study, 53 of these isolates were again screened for the *mcd* gene along with 75 new isolates from two French soils using a PCR-based assay (Desaint *et al.*, 2000). Sequences homologous to the *mcd* gene were only detected in 45% of the 128 isolates and both *mcd*-positive and *mcd*-negative strains could be isolated from the same soil. Similarly, Feng *et al.* (1997) failed to detect hybridization of five plasmids from a carbofuran-degrading *Sphingomonas* sp. strain CF06 with the *mcd* gene probe. Interestingly, further investigation with this strain found that carbofuran-degrading activity was lost when all five plasmids were cured and that a *Pseudomonas fluorescens* strain gained carbofuran-degrading ability when the five plasmids were transferred via conjugation. They also reported the presence of several insertion sequence (IS) elements that hybridised with some of the plasmids. Investigating this further, Ogram *et al.* (2000) reported the presence of sequences that hybridised with the sequence of an IS element from strain CF06 on similar-sized plasmids from two *Sphingomonas* sp. isolates originating from a different region of America to CF06. The genetic transfer of the *mcd* gene amongst soil bacterial populations has also been reported by Desaint *et al.* (2003). These studies demonstrate the importance of ECEs in recruiting genes from other bacteria to allow metabolism of new compounds. Gene transfer between related and unrelated bacteria may also explain the high degree of genetic diversity observed amongst carbamate-degraders (Desaint *et al.*, 2003)

Genes involved in the hydrolysis of the organophosphate parathion and the herbicide atrazine have also been identified and been found to be present in bacteria from different geographical locations. Serdar *et al.* (1982) identified a gene responsible for parathion hydrolysis located on a plasmid from a parathion-degrading *P. diminuta* strain. This organophosphate-degrading gene, the *opd* gene, has been found in other isolates capable of degrading parathion. Mulbry *et al.* (1986) demonstrated that a Philippine isolate,

Flavobacterium sp. (ATCC 27551), possessed sequences homologous to the *opd* gene on a 43 kb plasmid, although the plasmid was not homologous to that in *P.diminuta*. Homology with the *opd* gene was also investigated by Chaudhry *et al.* (1988b) who found that a *Pseudomonas* sp. capable of degrading parathion hybridised with an *opd* gene probe from *Flavobacterium* sp (ATCC 27551). The *opd* gene is not always found in organophosphate-degrading bacteria; for example, Singh *et al.* (2003a) failed to detect it in a chlorpyrifos degrader or chlorpyrifos-degrading soils.

The genes *atz*A, B and C that encode atrazine hydrolase have been reported to be widely distributed among atrazine-degraders (De Souza *et al.*, 1998; Rousseaux *et al.*, 2001; Ostrofsky *et al.*, 2002; Radosevich and Tuovinen, 2004). Rousseaux *et al.* (2002) investigated the occurrence of the *atz*ABC genes in 25 atrazine-degrading bacteria isolated from various French soils and found all isolates to contain at least one of the genes. They also detected similar IS elements on plasmids from all isolates carrying the *atz*BC genes.

As the above studies have shown, not all carbamate-degrading bacteria contain the *mcd* gene leading to the assumption that a number of genes are involved and have yet to be identified. Hashimoto *et al.* (2002) identified a novel carbaryl hydrolase gene (*ceh*A) in a *Rhizobium* sp. Additional genes involved in the degradation of organophosphates have also been identified. Zhongli *et al.* (2001) isolated a methyl parathion-degrading gene (*mpd* gene) from a methyl parathion-degrading *Plesiomonas* sp that is distinct from the *opd* gene. In both cases the genes were located on plasmids.

It should be noted that although enrichment culture experiments are of immense value to the study of pesticide-degrading bacteria, there is evidence that repeated subculture during enrichment procedures can, as a result of gene transfer between bacteria, lead to changes in the nature of the bacteria involved in the degradation (Fernandez *et al.*, 1999; Newby *et al.*,

2000; Desaint *et al.*, 2003). There is a possibility that the enrichment culture method leads to the isolation of bacteria capable of rapid degradation of the pesticide but which may not actually be major contributors to enhanced degradation in the field. However, Bending *et al.* (2003) have demonstrated that their isoproturon-degrading isolates (*Sphingomonas* spp.) were involved in the enhanced degradation observed in the field. Similarly, Singh *et al.* (2003a) found evidence that a chlorpyrifos degrader isolated from an Australian soil was most likely involved in the degradation in the soil.

1.5.3 Cross-Enhancement

The phenomenon of enhanced degradation becomes an even bigger problem when, as research demonstrates, a nematicide can be rapidly degraded in a soil that has no previous history of treatment with that chemical but has instead been treated with pesticides from the same chemical group. It is thought that as a result of the similarity of structurally related chemicals, such as carbamates, one pesticide may induce enzymes capable of degrading a chemically related compound (Kearney and Kellogg, 1985). Morel-Chevillet *et al.* (1996) demonstrated the cross adaptation effects amongst 15 carbamate pesticides. Pre-treatments with any of the 15 carbamates resulted in enhanced degradation of carbofuran. A good correlation between an increase in the size of the carbofuran-degrading population after pre-treatment with the variety of chemicals and subsequent enhanced degradation of carbofuran was observed, demonstrating that soil microorganisms were capable of degrading a variety of carbamate compounds. In contrast however, Racke and Coats (1988b) investigated the degradation of carbofuran, bendiocarb, carbaryl and cloethocarb in soils previously treated with either carbofuran, a mixture of carbamates, or cloethocarb and found that not all the carbamates tested were degraded as rapidly as carbofuran in enhanced carbofuran-degrading soils. Bendiocarb, a carbamate of similar structure to carbofuran, was rapidly degraded; however, the degradation of carbaryl and cloethocarb was most rapid in soils pre-treated with carbaryl or cloethocarb. This suggests

that the rate of hydrolysis may depend on the degree of similarity of the chemical structure to carbofuran. The authors hypothesised that different populations of carbofuran-degrading microorganisms were induced depending on the range of substrates that the soil had previously been exposed to. Of interest is the study by Suett and Jukes (1988) in which different areas of the same field received a previous treatment of aldicarb, thiofanox or carbofuran. The degradation rate of aldicarb was faster in samples taken from the carbofuran-treated area than in the aldicarb or thiofanox previously treated samples. In this soil the effects of cross-enhancement appeared to be greater than those of self-enhancement.

Individual carbofuran-degrading bacterial isolates have frequently been found to degrade other carbamates when examined in liquid enrichment culture. Parekh *et al.* (1995) isolated 55 bacteria capable of hydrolysing carbofuran and, of those, 50 were also capable of degrading carbaryl, aldicarb and methylamine. A number of the organisms listed in Table 3 also demonstrate degradation capability for other similarly structured chemicals in addition to the initial pesticide for which they were isolated.

Cross-enhancement of organophosphorus compounds appears to be less common than the cross enhancement seen with carbamates. For example, Racke and Coats (1988a) reported that neither chlorpyrifos, fonofos, ethoprop, terbufos or phorate were rapidly degraded in a soil that exhibited enhanced degradation of isofenphos. Similarly, soils with a history of fonofos treatment only enhanced the degradation of fonofos and none of the other organophosphates tested. Also, Smelt *et al.* (1996) found no evidence for enhanced degradation of ethoprophos in soils previously treated with fenamiphos and vice versa. In their recent study, Singh *et al.* (2005) reported the cross enhancement of parathion, diazinon and coumaphos in a soil that had demonstrated enhanced chlorpyrifos degradation in the laboratory. Cross-enhancement of organophosphorus nematicides and insecticides in

an enhanced fenamiphos-degrading soil was, however, not reported. The authors concluded that, like the carbamates, the degree of structural similarlity between compounds may explain why cross-enhancement was observed between chlorpyrifos and the other organophosphates tested but not in the enhanced fenamiphos-degrading soil.

The specificity of cross-enhancement for chemicals of similar structure is strongly evident between carbamates and organophosphates for which few, if any, incidences of cross-enhancement have been reported. Smelt *et al.* (1987) reported the rapid transformation of oxamyl in soils previously treated with aldicarb and vice versa, indicating the occurrence of cross-enhancement between the two carbamates. However, previous treatment with the organophosphate ethoprophos did not result in rapid degradation of oxamyl or aldicarb. Similarly, in a later study Smelt *et al.* (1996) reported the lack of cross-enhancement of oxamyl when incubated in a soil previously treated with ethoprophos. In a recent investigation into the biodegradation of the nematicide cadusafos, Karpouzas *et al.* (2004) reported the cross-enhancement of a cadusafos-degrading soil to rapidly degrade the similarly structured chemical ethoprophos but not the carbamate oxamyl. The absence of cross-enhancement between carbamates and organophosphates provides a useful strategy for preventing the development of enhanced degradation, in which carbamates and organophosphates are alternated within the crop rotation.

1.5.4 Transferability of Laboratory Data to the Field

The majority of enhanced degradation investigations are carried out in laboratory incubation studies under constant temperature and moisture conditions and, although laboratory incubation studies are a valuable method for establishing the degradation potential of soils, these abiotic conditions are rarely achieved in the field situation. It should not be assumed therefore that enhanced degradation as observed in the laboratory always implies imminent failure of the pesticide in the field. Suett and Jukes (1988)

reported enhanced degradation of aldicarb in a soil that had previously only received one application in the field. The enhanced degradation observed in the laboratory incubation was not mirrored in the field as no reduction in effectiveness to control cabbage root fly had previously been measured in this field (Suett, 1987). Similarly, after 20 years of annual applications to an experimental field plot the rate of hydrolysis of aldicarb sulphoxide and aldicarb sulphone was found to be greatly enhanced when investigated in a laboratory incubation study. However, aldicarb was reported to still be suppressing plant-parasitic nematode populations after 18 years of annual treatment (Bromilow *et al.*, 1996).

Of course, there are also contrasting studies in which enhanced degradation measured in the laboratory has also been reported in the field, e.g. Felsot *et al.* (1982); Suett (1987) and Suett *et al.* (1993). Suett (1996b) reported the control of cabbage root fly larvae by carbofuran to be worse in all previously treated soils when compared to the previously untreated controls. This corresponded to the much faster degradation rates in the previously treated samples observed in the laboratory. Similar results were obtained with chlorfenvinphos, although results were much less marked in soil samples of pH 6.3 or less. Previously untreated samples gave greater larvae control in the field and demonstrated slower degradation rates in the laboratory.

1.5.5 Analysing Pesticide Degradation Data

Quantitative comparisons of differences between treatments in laboratory incubation experiments are necessary in order to fully analyse and interpret degradation data. The time taken for the initial application of the chemical to degrade to 50%, known as the DT50 or half-life, is a common ground frequently used to assess treatment differences (Walker *et al.*, 1993; Smelt *et al.*, 1996; Karpouzas *et al.*, 2001; Walker *et al.*, 2001). Differences between the DT50 values can then be statistically analysed using a simple Analysis of Variance (Singh *et al.*, 2002; Sorensen and Aamand, 2003). Laboratory degradation data is

often assumed to conform to first-order rate kinetics because abiotic factors, such as soil moisture and temperature, are, to a certain extent, controlled and therefore not a limiting factor. As a result, the rate-limiting component is assumed to be the concentration of the pesticide, *i.e.*, an exponential relationship exists between the amount of pesticide dissapearing per unit time and pesticide concentration. A plot of the log of concentration against time gives a straight line (Hamaker, 1972; Walker, 1987; Alexander, 1994). Linear regression of the straight line yields a slope value that is proportional to the rate constant and from this the time taken to 50% degradation, referred to as the first-order half-life, can be calculated (Walker, 1987). However, degradation data does not always conform to first-order kinetics (Morel-Chevillet *et al.*, 1996; Karpouzas *et al.*, 2001). In enhanced degradation studie, pesticide degradation has also been seen to increase with time (Karpouzas *et al.*, 2001; Bending *et al.*, 2003). This is particularly true of pesticide degradation within liquid enrichment cultures, *e.g.*, Chaudhry and Ali, 1988; Cox *et al.*, 1996; Karpouzas *et al.*, 2000a; Neumann *et al.*, 2004. A lag phase, during which bacteria are adapting in some way in order to degrade the pesticide, may be evident prior to a more rapid phase of degradation (Kearney and Kellogg, 1985). As such, plotting the log of concentration against time does not give a straight line and therefore can not be analysed by linear regression. In cases like these researchers use non-linear curves, such as the Gompertz curve, fitted to untransformed data to describe the pattern of degradation and to calculate the DT50 value (Morel-Chevillet *et al.*, 1996; Karpouzas *et al.*, 2001; Martin-Laurent *et al.*, 2004). In addition, there can often be insufficient data to accurately determine if the degradation is first-order. Sorenson and Aamand (2003) make a point of mentioning that a minimum of four data points were used for their linear regression analysis. However, Hamaker (1972) suggests that in order to gain a realistic fit of the data to the equation at least nine to 15 data points should be used. A drawback of calculating a DT50 value instead of a first-order half-life is that the DT50 value is not independent of concentration and as such can not be used to predict the disappearance time of

concentrations that were not measured in the experiment (Hamaker *et al.*, 1972). Degradation data are often interpreted without the use of statistical analysis, *e.g.*, Cox *et al.* (1996); Karpouzas and Walker (2000b); Rouseaux *et al.* (2001); Karpouzas *et al.* (2004). This is possibly because of the complex nature of decay curves and also because differences between estimated DT50 values can be very large, such as those reported for the soils tested by Suett (1986) and Smelt *et al.* (1996).

1.6 MANAGING ENHANCED DEGRADATION

1.6.1 Prevention and Cure

It may be possible to reduce the degrading capacity of an enhanced degrading soil with the use of soil sterilants, or to prevent it from occurring through the use of crop rotation and/or chemical rotation (alternating between pesticides of different chemical groups e.g., carbamates and organophosphates) (Section 1.5.3). The combined use of a fumigant nematicide prior to the application of a granular nematicide has been shown to reduce the activity of pesticide-degrading soil microorganisms in soils that have developed an enhanced degradation problem. Karpouzas *et al.* (2005a) investigated this possibility with the fumigants metham sodium and methyl bromide and the non-fumigant nematicide cadusafos. Application of methyl bromide to a greenhouse soil 9 months prior to analysis for cadusafos degradation in a laboratory incubation study failed to reduce the rate of cadusafos degradation. In contrast, the inhibitory effect of metham sodium remained throughout the 9 months, allowing cadusafos to persist in the soil resulting in high root-knot nematode mortality. When either fumigant was applied to the soil samples 20 days prior to cadusafos addition, both methyl bromide and metham sodium were effective in inhibiting rapid cadusafos degradation. Both fumigants were found to have significantly reduced the size of the soil microbial biomass. The authors concluded that the combined use of metham sodium and cadusafos could prevent the development of enhanced degradation of cadusafos. Similarly, in a study by Suett (1986) in which previously treated

soils were tested for enhanced degradation of carbofuran, one of the soils that had been treated with the soil sterilant dazomet for 5 years running, in addition to annual applications of carbofuran, failed to exhibit enhanced degradation. This indicated that dazomet effectively reduced the degrading activity of the soil microorganisms. This was investigated further in Suett (1987); however conversely in this study dazomet failed to improve the performance of carbofuran. The author put these variable results down to differences in dazomet application between the two studies. The 1986 experiment was covered with plastic sheeting after dazomet application, retaining the chemical well, whereas the 1987 study did not receive this treatment. One problem with the use of soil fumigants such as 1,3-D is that they too are susceptible to enhanced degradation when used repeatedly (Section 1.4.3.3).

Studies have shown that the enhanced degrading ability of a soil can remain for a number of years after application of the pesticide, suggesting that for some pesticides a lengthy crop rotation, where the chemical is applied less frequently, may be needed to prevent enhanced degradation occurring. Smelt *et al.* (1996) monitored degradation rates of the nematicides ethoprophos, aldicarb and oxamyl in a soil that had received in-field treatment with all 3 chemicals 5 years previously. The degradation rates of aldicarb and oxamyl, although slightly slower than the degradation rate 5 years ago, were still much faster than that of the previously untreated control; the degradation rate of ethoprophos was no different from the control. Similarly, in a laboratory incubation study, Karpouzas *et al.* (2001) found that 12 months after an initial treatment with a low carbofuran dose of 0.1 mg kg^{-1} the top-soil microbes were still degrading carbofuran at a faster rate than the initially untreated control. This supports findings from their earlier study (Karpouzas *et al.*, 1999a) where one initial in-field carbofuran application initiated enhanced rates of carbofuran degradation that lasted for the next 4 years. A lengthy crop rotation to prevent enhanced

degradation fits in well with PCN integrated management techniques where crop rotations of 7-8 years are recommended to reduce PCN population densities (Section 1.3.2.1)

1.6.2 Identifying Problem Soils

Molecular tools, such as using specific degradation genes as probes e.g. the *mcd* gene or the *atz* genes, have been suggested for predicting the occurrence of enhanced degradation. However, these all have their drawbacks. As mentioned in Section 1.5.2, the *mcd* gene is not present in all bacteria able to degrade carbamates and other carbamate hydrolase genes have yet to be identified. Also, although a number of the genes involved in atrazine hydrolysis have been identified and found to be widespread amongst atrazine degraders, it does not always follow that a soil exhibiting high genetic potential for atrazine degradation will rapidly degrade atrazine. Using quantitative PCR of the *atz*ABC genes, Martin-Laurent *et al.* (2004) measured the atrazine-degrading potential of three French soils. The soil demonstrating the highest rate of atrazine degradation also possessed the highest density of *atz* gene sequences; however the remaining two soils exhibited similar atrazine-degrading genetic potential despite differing atrazine mineralization rates. The authors suggested that the expression of atrazine-degrading genetic potential was affected by soil physicochemical properties, such as the high clay content of one soil which possibly reduced the bioavailability of atrazine. If soil characteristics are taken into consideration however, the use of quantification methods such as quantitative competitive PCR to determine the frequency of specific degradation genes, particularly the *atz* genes, may be of use in predicting enhanced degradation. In addition to their above mentioned study, Martin-Laurent *et al.* (2003) reported that the quantity of *atz*C gene increased dramatically in a previously treated soil after treatment with atrazine. This corresponded to a more rapid rate of degradation seen in this soil in comparison to the previously untreated sample.

Quantification of specific bacterial species that have previously been identified as carbamate degraders may give an indication of a soil's potential for enhanced degradation.

This would however only be effective in areas where it has been shown that a particular species is the main instigator of enhanced degradation, as the high degree of genetic diversity amongst methylcarbamate degraders has already been established (Table 3).

1.7 RESEARCH OBJECTIVES

The research detailed in this thesis was designed to investigate the enhanced degradation of non-fumigant nematicides and possible assays for predicting the enhanced degradation of nematicides in soils.

Experiments were designed to test the following hypotheses:

- There is potential for enhanced degradation of the carbamate nematicides aldicarb and oxamyl and the organophosphate fosthiazate within UK potato fields.

- The rate of degradation of the nematicides aldicarb, oxamyl and fosthiazate in soils increases with successive applications, demonstrating the involvement of adapted soil microorganisms.

- Bacteria capable of rapidly degrading oxamyl can be isolated from enhanced degrading soils.

- Similar bacterial species involved in the degradation of oxamyl can be isolated from different soils.

- Bacterial isolates capable of oxamyl degradation harbour DNA sequences homologous to the *mcd* gene.

- The ability to degrade oxamyl is easily lost, implying the involvement of plasmids.

- The enrichment culture method is a useful and reliable tool for predicting enhanced degradation in field soils.

2. ENHANCED DEGRADATION OF THE NEMATICIDES ALDICARB, OXAMYL AND FOSTHIAZATE IN UK AGRICULTURAL SOILS

2.1 INTRODUCTION

One of the main tools available to control potato cyst nematodes in the UK are soil-applied, granular nematicides; the oximecarbamates aldicarb and oxamyl, and the organophosphate fosthiazate are the main granular nematicides in use in the UK (Evans and Haydock, 2000; Garthwaite *et al.*, 2002). Within soil, aldicarb is largely converted by oxidation to its toxic oxidation products aldicarb sulphoxide and aldicarb sulphone. Toxicity is lost from all three compounds via hydrolysis of the methylcarbamate linkage (Bromilow *et al.*, 1980, Jones and Norris, 1998). Oxamyl is less susceptible to oxidation and instead is hydrolysed to its non-toxic oximino metabolite (Bromilow, 1973; Harvey and Han, 1978). Degradation of fosthiazate has been little researched; however, it has been suggested that due to its chemical structure it may, like fenamiphos, be converted via oxidation to toxic sulphoxides and sulphones (Qin *et al.*, 2004).

The enhanced degradation of carbamate and organophosphorus nematicides-insecticides, including aldicarb and oxamyl, has previously been reported (Felsot *et al.*, 1982; Smelt, 1987; Suett, 1987; Karpouzas *et al.*, 1999a). Ambrose *et al.* (2000) reported the rapid disappearance of oxamyl residues from a field site in Shropshire, England, where the chemical could not be detected 21 days after application. As such, there is concern over the potential for enhanced degradation of granular nematicides in UK agricultural soils. Poor nematode control by nematicides can be due to various factors. However, when granular-soil incorporation technique has been optimal and the nematicides have been incorporated to the correct depth (Woods and Haydock, 2000), reduced efficacy could well be due to enhanced rates of degradation within the soil. Biotic and abiotic factors are involved in the

degradation of pesticides within the soil (Smelt *et al.*, 1979; Bromilow *et al.*, 1980; Smelt, 1987). However, it is biotic factors, namely soil microorganisms, that are responsible for enhanced degradation. With some pesticides, repeated application at the same site can result in the adaptation of soil bacteria capable of utilising the chemical as an energy source, resulting in the enhanced degradation of that chemical (Suett, 1986; Smelt *et al.*, 1987; Ou *et al.*, 1994; Karpouzas, 1999a; Sorensen and Aamand, 2004; Karpouzas *et al.*, 2004). This has also been observed in the laboratory where the rate of degradation is often seen to increase with subsequent applications, providing evidence for the presence of adapted bacteria and therefore the potential for enhanced degradation in the field (Read, 1987; Cox *et al.*, 1996). As such, soil samples collected from different potato growing fields around England were screened for their potential for enhanced degradation by applying three repeat applications of aldicarb, oxamyl or fosthiazate in the laboratory.

2.1.1 Objectives

- To determine the potential for enhanced degradation of the nematicides aldicarb, oxamyl and fosthiazate in geographically distinct agricultural soils taken from different locations within theUK.

- To attempt to induce enhanced degradation of aldicarb, oxamyl and fosthiazate by applying three successive nematicide applications within the laboratory so that, ultimately, bacteria involved in enhanced degradation could be isolated from these soils.

2.2 MATERIALS AND METHODS

2.2.1 Obtaining the Soil Samples

Initially an advertising campaign was conducted to appeal to farmers who may have experienced poor performance of a granular nematicide. Articles were posted in major farming publications, such as Farmer's Guardian, Potato Review and The Farmer. However, due to a poor response, possibly due to the difficulty of pinpointing the cause of nematicide failure, a different approach had to be taken.

By co-ordinating with a colleague, farmers from the major potato-growing areas of England, who had grown potatoes in the previous year, were contacted and visits made to collect soil samples. Soil samples were taken from fields in Yorkshire, Lancashire, Norfolk, Cambridgeshire, Kent, Sussex, Shropshire, Devon and Cornwall in 2001. Thirty-five samples were collected in total. At each farm, nematicide application history and rate information was collected.

2.2.1.1 Soil Sampling

To prevent cross contamination of soil microorganisms between soil samples, a sampling protocol was developed. This involved using a spade that had been welded along the spade head and handle to fill in large cracks that could trap soil. The spade head was soaked in 1% Virkon solution (a broad-spectrum disinfectant) for at least ten minutes prior to sampling in order to kill off microorganisms, and wiped clean of soil after sampling.

Each sample was taken from one place within a field and the position was recorded using Global Positioning System (GPS) to enable further soil to be taken from the original sampling site if more soil was required at a later date. Approximately 5 kg of soil was collected at each location and the samples were kept chilled in an icebox during transit.

The samples were kept at 4°C before being sieved through a 5.6 mm mesh to remove stones and large organic material. No sample was chilled for more than one week. If necessary, soils were air-dried at room temperature to below 70% of their available water capacity (AWC) prior to sieving. To prevent cross contamination between samples, sieves were soaked in 1% Virkon solution for at least 15 min before and after sieving. Samples were mixed by shaking vigorously, then divided into approximately 1 kg portions and stored at – 20 °C.

2.2.2 Soil Property Analysis

2.2.2.1 Particle-Size Analysis

The ADAS method (Anon, 1986) was used to examine the particle-size distribution of the soil samples. Ten grams of the <2 mm fraction of air-dried soil was placed into a 600-ml beaker, to which 10 ml of 30% hydrogen peroxide solution was added. If a vigorous reaction occurred, additional 10 ml amounts of 30% hydrogen peroxide were added. The soil suspensions were then left to stand over night.

The beakers were topped up to approximately 40 ml with hydrogen peroxide and gently heated on a hot plate until the organic matter had been oxidised. They were then allowed to cool before being transferred to 250-ml shaking bottles containing 10 ml of dispersing reagent (50 g sodium hexametaphosphate and 7 g anhydrous sodium carbonate in 1 l) and 150 ml of water, and shaken for 5 min. The solution was then poured through a 63-mm sieve into a 500-ml graduated cylinder. The residue remaining on the sieve was thoroughly washed to ensure all small particles passed through. The contents of the sieve were transferred to an evaporating dish and dried in an oven at 105°C over-night.

The graduated cylinder was topped up to 500 ml and shaken by inverting 20 times. A 25 ml sample was immediately taken from a depth of 100-150 mm below the surface and

transferred to an evaporating basin for drying at 105°C. This was the < 63 μm fraction. The cylinder was then left to stand for 7 h to allow the suspension to equilibrate. After 7 h, a 25 ml sample was taken from 90 mm below the surface and transferred to an evaporating basin for drying at 105°C, this was the < 2 μm fraction.

The sand (> 63μm fraction), silt (< 63μm) and clay (< 2 μm) fractions were weighed after drying and their percentages calculated. The soil texture type was determined using a texture triangle (Rowell, 1994). Soil particle analysis for each soil are displayed in Table 4.

2.2.2.2 Organic Matter Content

Ten grams of air-dried and sieved soil (< 2 mm mesh) were weighed into a pre-weighed crucible and placed in a drying oven at 105°C over-night. The oven-dried soil was allowed to cool in a dessicator prior to weighing to give the moisture content of the soil. The crucible was then transferred to the furnace where it was ignited at 500°C for 18 h. After cooling in a dessicator, the sample was re-weighed to give the organic matter content.

2.2.2.3 pH

Ten grams of air-dried, sieved soil were added to a 50-ml plastic, screw-cap container along with 25 ml of de-ionised water. The container was capped and shaken vigorously by hand. The pH of the soil suspension was measured using a Russell RL150 pH meter.

2.2.2.4 Available Water Capacity

Soil samples were to be adjusted to 70% of their available water capacity (AWC) so that soil moisture content would not have an effect on the development of enhanced degradation. Soil Survey mean available water capacity values based on soil type (Hall *et al.*, 1977) were used to determine the AWC of the soil samples. Seventy percent of the mean AWC value was calculated. The AWC of peat soils was based on information

received via a personal communication from John Hollis (Principal Research Scientist, National Soil Resources Institute).

2.2.3 Nematicide Concentration Analysis

2.2.3.1 Calibration of the HPLC

Measurement of chemical concentrations by High Pressure Liquid Chromatography (HPLC) requires a calibration curve to be incorporated into the method so that the area of the chemical peak on the chromatogram can be converted into parts per million (ppm) of chemical concentration. Fifty parts per million standards were made up in 50:50 HPLC grade acetonitrile and HPLC grade water for fosthiazate and aldicarb and its oxidation products; and 50:50 HPLC grade methanol and water for oxamyl. The 50 ppm standards were then further diluted in the relevant solvent mix to give standards of 5, 2.5, 1.5, 1.0 and 0.5 ppm, to cover the range of concentrations expected to be found in the soil samples. Standards were then analysed by HPLC following the relevant method for each nematicide (Section 2.2.3.2) and the calibration curves established. Calibration curves for aldicarb, aldicarb sulphoxide and sulphone were determined using standards that were a mixture of all three compounds. Analytical grade oxamyl, aldicarb, aldicarb sulphoxide and aldicarb sulphone were obtained from Greyhound Chemical Suppliers and analytical grade fosthiazate was obtained from Syngenta.

2.2.3.2 Nematicide Analysis by HPLC

All chemical standards and soil extractions were analysed using a Hewlett Packard Series 1100 HPLC. Analysis of aldicarb concentrations required the use of a Merck Lichrocart Lichrosorb column-RP-18 (17 μm) held at 30°C. The mobile phase was run at a gradient commencing with 10% acetonitrile and 90% water, running to 100% acetonitrile and no water over 8 min with a 3 min delay between each injection. The solvent ran at a flow rate

of 1.3 ml/min and a 20 µl injection was monitored at 210 nm with a retention time of 2.93 min for sulphoxide, 3.34 min for sulphone and 5.19 min for parent aldicarb.

Table 4: Soil property analysis of incubation study soils treated with aldicarb (ALD), fosthiazate (FOS) or oxamyl (OX).

Location	*Soil*	*% Sand*	*% Silt*	*% Clay*	*Soil Type*	*% OM*	*pH*	**Moisture content (%)[a]**
Cheshire	ALD 1	50	30	20	clay loam	6	6.7	15.5
Southport, Lancashire	ALD 2	30	57	13	Peat	70	5.8	29.4
Lydiate, Lancashire	ALD 3	10	51	39	Peat	52	5.8	29.4
Newport, Shropshire	ALD 4	74	15	11	Sandy loam	3	6.6	12.9
Telford, Shropshire	ALD 5	8	42	50	Loamy peat	36	5.5	22.4
Lincolnshire	ALD 6	55	19	26	Sandy clay loam	6	7.6	14.0
Shifnal, Shropshire	ALD 7	74	17	9	Sandy loam	3	6.3	12.9
Rugby, Warwickshire	ALD 8	67	18	15	Sandy loam	6	5.6	12.9
Rugby, Warwickshire	ALD 9	56	28	16	Sandy loam	6	5.8	12.9
Rugby, Warwickshire	ALD 10	55	27	18	Sandy loam	6	6.5	12.9
Yorkshire	ALD 11	81	8	11	Sandy loam	4	7.3	12.9
Yorkshire	ALD 12	66	24	10	Sandy loam	7	6.7	12.9
Yorkshire	ALD 13	69	19	12	Sandy loam	3	6.3	12.9
St Nicholas, Kent	ALD 14	26	50	24	Clay loam	6	7.5	15.5
Gravesend, Kent	ALD 15	47	30	23	Clay loam	6	7.3	15.5
Lydiate, Lancashire	FOS 1	38	41	21	Peaty loam	30	5.9	22.4
Newport, Shropshire	FOS 2	73	15	12	Sandy loam	3	6.1	12.9
Waddingham, Lincolnshire	FOS 3	69	15	16	Sandy loam	4	7.6	12.9
Shifnal, Shropshire	FOS 4	65	30	5	Sandy loam	3	6.6	12.9
Cambridgeshire	FOS 5	43	47	10	Sandy silt loam	6	7.5	15.4
Chestnut Farm, Cambridgeshire	FOS 6	53	34	13	Sandy loam	5	7.5	12.9
Kings Fm, Cambridgeshire	FOS 7	35	48	17	Peaty loam	35	7.0	22.4
Kings Fm, Cambridgeshire	FOS 8	30	41	29	Peaty loam	30	6.3	22.4
Kings Fm, Cambridgeshire	FOS 9	45	31	24	Clay loam	20	7.3	15.5
Kings Fm, Cambridgeshire	FOS 10	30	50	20	Loamy peat	40	6.7	22.4
Owmby by Spittal, Lincolnshire	OX 2	66	11	23	Sandy clay loam	5	7.5	14.0
Blaco Hill Fm, Lincolnshire	OX 3	82	11	7	Loamy sand	3	6.7	9.4
Glentworth, Lincolnshire	OX 4	56	17	27	Sandy clay loam	6	7.6	14.0
Cromer, Norfolk	OX 5	35	54	11	Sandy silt loam	4	5.9	15.4
Cromer, Norfolk.	OX 7	38	49	13	Sandy silt loam	4	7.2	15.4
Ely, Cambridgeshire	OX 8	9	46	45	Peat	70	6.4	29.4
Penzance, Cornwall	OX 9	46	46	8	Sandy silt loam	7	7.0	15.4
Penzance, Cornwall	OX 10	61	28	11	Sandy loam	4	7.2	12.9
Braunton, Devon	OX 11	50	33	17	Clay loam	5	7.4	15.5
Fareham, Hampshire	OX 12	22	50	28	Clay loam	4	6.2	15.5

[a] 70% of published AWC values (Hall *et al.*, 1977)

Prior to HPLC analysis, all oxamyl extracts had to be diluted 50:50 with HPLC grade water to prevent some of the oxamyl from travelling through the column at a faster rate than the rest of the sample, resulting in inaccurate measurements of oxamyl concentration. Oxamyl analysis was carried out using a Phenomenox, 250 x 46 mm Sphereclone 5 m ODS column held at 30°C. The mobile phase was 50:50 HPLC grade water and methanol run at a flow rate of 1.2 ml min^{-1}. A 20 µl injection was monitored at 220 nm with a retention time of 3.5 min. Both aldicarb and oxamyl analysis closely followed the method used by Ambrose *et al.* (2000)

Fosthiazate analysis followed the method used by Woods and Haydock (1999b). A Phenomenox, 250 x 46 mm Sphereclone 5 µm ODS column held at 30°C was used with a mobile phase of 50:50 acetonitrile and water run at a flow rate of 1.0 ml min^{-1}. The 20 μl injection was monitored at 230 nm with a retention time of 7.4 min.

2.2.3.3 Analytical Efficiency of the Extraction Procedure

Prior to commencing the incubation study, a preliminary investigation was carried out to determine how much aldicarb, oxamyl or fosthiazate was recoverable using the extraction procedure described in Section 2.2.3.4. The extraction procedure was tested on a number of soil types with all three nematicides.

Twenty grams of air-dried, sieved soil (< 2 mm mesh) were weighed into a 100-ml, glass-shaking bottle and spiked with 1.0 ml of a 50 mg l^{-1} standard to give 2.5 mg l^{-1} of either oxamyl or fosthiazate. To determine the analytical efficiency of the extraction procedure for aldicarb and its two oxidation products, aldicarb sulphoxide and aldicarb sulphone, 1.0 ml of a 50 mg l^{-1} standard of each was added to the soil. All soils were thoroughly mixed by vigorous shaking before being allowed to equilibrate for 30 min. Methanol (20 ml) was added to the soil samples and shaken on a reciprocating shaker at 300 rpm for 3 h. The

supernatant was then removed using a 2-ml syringe and cleaned by passing through a 0.45-μm syringe filter. Extracts were then analysed by HPLC (Section 2.2.3.2).

Initially, recovery rates for oxamyl were low because the high concentration of methanol passing into the HPLC column with the sample injection caused some of the oxamyl to travel through the column faster than the rest of the sample. This resulted in a shoulder on the front of the peak on the chromatogram causing inaccurate measurements. However, after a number of tests to try to eliminate the problem it was found that by diluting the extract with HPLC water at a ratio of 1:1 the recovery rates improved. Using the diluted extract, recovery rates exceeded 80% in a peat soil and were greater than 90% in a sandy loam.

Fosthiazate gave recovery rates of more than 90% in a peat soil and more than 80% in a sandy loam however, recovery rates were only 50% in a sandy clay loam and a clay loam. Only one of the soils tested for fosthiazate degradation was a clay loam, so the low recovery rates should not have been a major problem for the experiment. Recovery rates of aldicarb, aldicarb sulphoxide and aldicarb sulphone exceeded 90% in sandy loam, clay loam and peat soils.

The limits of detection of the HPLC methods were confirmed down to a low concentration of 0.02 $\mu g\ ml^{-1}$ using standards for aldicarb, aldicarb sulphoxide, oxamyl and fosthiazate; and to a concentration of 0.5 $\mu g\ ml^{-1}$ for aldicarb sulphone.

2.2.3.4 Nematicide Extraction from Soils

Aldicarb, oxamyl and fosthiazate were all extracted from the 20 g soil samples by shaking with 20 ml of HPLC grade methanol for three hours on a reciprocating shaker set at 300 rpm. The supernatant was filtered through a 0.45-μm cellulose acetate syringe filter into

1.5-ml Eppendorf tubes. Extracts were stored at –20°C before analysis by HPLC (Section 2.2.3.2).

2.2.4 Incubation Study

The incubation study method closely followed that by Cox *et al.* (1996). Soils to be used in the incubation study were removed from the freezer, where they had been stored at –20°C since sampling, and left to defrost over-night at room temperature. Soil was weighed into plastic bags to the equivalent of 1.8 kg dry weight soil and the soil moisture content adjusted to 70% of AWC by adding sterile distilled water until the soil reached its correct weight. The soils were then mixed thoroughly by vigorous shaking. The soil was then distributed between six, 500-ml screw-cap, polyethylene containers with 300 g of dry weight soil added to each. The containers were labelled as first, second or third dose, with two replicates for each. All samples were then left at room temperature over-night.

The nematicide applied to the soil samples, aldicarb, oxamyl or fosthiazate, was the same as the last nematicide that had been incorporated into the soil in the field. This resulted in 15 samples receiving aldicarb, ten receiving oxamyl and ten receiving fosthiazate applications. The appropriate nematicide was added in a 5 ml aqueous solution to all first, second and third dose soils at a rate equivalent to full field rate (oxamyl, 5.5 kg ha^{-1}; aldicarb, 3.36 kg ha^{-1}; fosthiazate, 3.0 kg ha^{-1}). In all cases the nematicide was 99% pure analytical-grade chemical. Aldicarb, aldicarb sulphoxide, aldicarb sulphone and oxamyl were obtained from Greyhound Chemical Supplies and fosthiazate was obtained from Syngenta. Aldicarb was applied at a rate of 1.8 mg kg^{-1} by adding 5 ml of a 108 mg l^{-1} solution in sterile distilled water (SDW); oxamyl was applied as 5 ml of a 175 mg l^{-1} solution in SDW to give a rate of 2.9 mg kg^{-1}; and fosthiazate was applied as 5 ml of a 95 mg l^{-1} solution in SDW to give a rate of 1.6 mg kg^{-1}. After addition of the nematicide, all soil samples were thoroughly mixed by shaking vigorously, tumbling and stirring using a

different disposable spoon for each soil. All replicates were randomised within an incubator at 15°C and were left with the lids loosely screwed down. Moisture contents were maintained by the addition of sterile distilled water when necessary based on soil weight.

All first-dose replicates were sampled immediately after addition of the nematicide, and then at approximatly 5-day intervals. At each sampling point, 20 g of soil was weighed out into a 100-ml, glass bottle using disposable plastic spoons to prevent cross contamination. Samples were immediately extracted and all extracts were stored at –20°C until analysis by HPLC. If samples could not be extracted immediately, the 20 g sub-samples were stored at –20°C until extraction could be performed. Sub-samples were defrosted at 4°C to limit the activity of pesticide-degrading microorganisms prior to extraction.

After the first 25 days, the second dose of nematicide was applied to all second-dose and third-dose soils. The application procedure followed that described for the first dose. Second-dose soils were sampled immediately after application and then at approximatly 5-day intervals over the next 25 days. After this period, all third-dose soils received the third and final application of the relevant nematicide. Sub-samples were taken immediately after application and at approximatly 5-day intervals for the remaining 25 days. All sub-samples were analysed for oxamyl concentration following the method described for the first-dose soils.

2.2.5 Analysis of Results

The time to 50% degradation of the initial nematicide concentration (DT50) was used to summarise the degradation data for the soil incubation study. This value was calculated from curves showing degradation against time, fitted to the raw data for each replicate using SigmaPlot version 8.0. Linear, exponential or Gompertz curves were fitted

depending on which curve accounted for the greatest amount of variation and provided the highest r^2 value. The rationale behind the curve fitting followed the principle of parsimony, by which models with the fewest terms and maximum explanatory power were selected first. As such, if a linear regression did not give a good fit, *i.e.*, an r^2 value of 0.95 or more an exponential or Gompertz curve was fitted. In the majority of cases, linear or exponential curves gave the best fit. All curves were fitted using SigmaPlot version 8.0. Equations for the three curves are shown below:

Linear: $y = y_0 + ax$

Exponential: $y = y_0 + ae^{-bx}$

Gompertz: $y = y_0 + ae^{-e^{-\left(\frac{x-x_0}{b}\right)}}$

To calculate the DT50 value the Solve function in SigmaPlot version 8.0 was used to return an X axes value from a known Y axes value based on the fitted curve. Significant differences between applications were determined by one-way Analysis of Variance (ANOVA) of the DT50 values from all replicates for each application (n=2) using Genstat version 5.0. Significance was measured at the 95% and the 90% confidence limits (P<0.05 and 0.1).

2.3 RESULTS

2.3.1 Oxamyl

The degradation of successive applications of oxamyl in different agricultural soils is displayed in Figure 7. Mean ($n=2$) values are presented as a percentage of the initial day zero value for each application. It should be noted that due to experimental error, day zero results were not available for the first oxamyl application applied to soil OX8. As a result mean data points for the first application are a percentage of the day 5 concentration. Due to the erratic nature of the data in many cases, the data points are displayed with lines of best fit to show the pattern of degradation more clearly. The time taken for each application to be degraded to 50% of the initial concentration (DT50) is displayed in Table 6 along with the r^2 values for the fitted curves that were used to calculate the DT50. Due to the variability in the oxamyl degradation data, it was difficult, in some cases, to fit a curve to the data. The amount of variance accounted for by the fitted curve was well below 95% for five of the soil samples. In the case of soil OX11, the fitted curves for all three applications accounted for only 80% or less of the variation. The degree of accuracy of the DT50 values in these cases will therefore be reduced. Variability between replicates for some soils was also very high as can be seen from the standard error values in Table 6. A complete dataset for all three nematicides is also available in Chapter 7, Tables 7.1 to 7.3. Due to this and the small number of replicates, statistical analysis of the differences between the DT50 values of the three applications was difficult. As such, differences between the three applications were deemed as significant at the 90% confidence limit as well as the 95% limit.

It should also be noted that for all three nematicides, the DT50 values were not calculated as first-order half-lifes due to the difficulty of applying linear regression models to semi-log transformed degradation data for a number of the soils. As a result the DT50 values are not independent of the concentration, as a half-life would be, and therefore it can not be

assumed that the percentage of pesticide lost per unit time is constant. As such the DT50 value can not be used to predict the persistence time for concentrations that were not measured in the experiment, *i.e.*, DT50 should not be predicted if the experiment was stopped at the point of 30% degradation (Hamaker *et al.*, 1972). In cases where degradation has been slow and the point of 50% degradation was not reached before the end of the experiment (primarily the fosthiazate data), SigmaPlot version 8.0 calculated the DT50 value by extending the fitted curve beyond the final data point. These values have been included in Tables 6, 7 and 8; however, they will be subject to a degree of error.

The second or third oxamyl applications, or both, were degraded significantly ($P<0.1$ or 0.05) faster than the first application in five out of the ten soils tested (soils OX 2, 5, 8, 9 and 12), suggesting that enhanced degradation had occurred. Of the remaining five soils, although there was no significant difference between the first application and the subsequent applications, oxamyl degradation was still rapid in all except soil OX 11. Oxamyl degradation was rapid for all applications applied to soil OX 10. Degradation proceeded at such a fast rate in the second and third applications that a curve could not be fitted to the data and thus no DT50 values are available; however they would be less than 1 day. Although the mean DT50 values for soil OX 3 show degradation to have been slightly slower in the second and third applications (4.1 and 4.7 days respectively) than in the first application (2.1 days), degradation was still occurring rapidly in all three applications. In addition, the standard error for the mean DT50 values for the second and third applications show variability between replicates, resulting in less accurate DT50 values. Similarly, although the difference between the first application and the subsequent applications to soils OX 4 and OX 7 were not significantly different, degradation was still rapid, particularly in the second and third applications. Mean DT50 values of 1.2 and 1.7 days for the second and third applications respectively were recorded for soil OX 4; and 2.3 and 1.6 days respectively were recorded for soil OX 7, whereas the time taken to degrade 50%

of the first application was 4.4 and 7.4 days for OX 4 and OX 7 respectively. However, the standard error values of the DT50 value for these first oxamyl applications are high, possibly contributing to the lack of significant difference between first and subsequent applications. Soil OX 11 is the only soil to demonstrate comparatively slow degradation in all three applications. Differences between the DT50 values of the three applications are not significant although the mean DT50 values do suggest that degradation is faster in the second (14.9 days) and third (12.3 days) applications than in the first (21.9 days).

The pH values of the ten soils ranged from 5.9 in OX 5 to 7.6 in OX 4 (Table 4). The pH of the slower degrading soil, OX 11, was at the top end of the range with a pH of 7.4. Interestingly, OX 8 in which the second and third applications of oxamyl were degraded at a significantly ($P<0.1$) faster rate than the first, had an extremely high organic matter content of 70%. This soil did however have a history of oxamyl application every four or five years, going back as far as 1979. Similarly, soil OX 10, in which oxamyl degradation was rapid from the outset in all three applications, had been treated with oxamyl in the field twice in the previous two years. It was also surprising that soil OX 4 demonstrated rapid oxamyl degradation in the second and third applications as this soil had been fumigated with the soil sterilant 1,3-dichloropropene in 2000 (Table 5).

Table 5: Nematicide application history for incubation study soils

Soil	*Nematicide application history*	*Rate (kg ai/ha)*
Ald 1	aldicarb	3.36
Ald 2	aldicarb	1.7
Ald 3	aldicarb	3.36
Ald 4	aldicarb 2001 oxamyl 1997 & 1993	aldicarb- 3.36
Ald 5	aldicarb 2001 & 1994	3.36
Ald 6	aldicarb 2001, 1,3-dichloropropene 2001	aldicarb- 3.36
Ald 7	aldicarb 2001	3.36
Ald 8	aldicarb 2001	3.36
Ald 9	aldicarb 2001	3.36
Ald 10	aldicarb 2001	3.36
Ald 11	aldicarb 2001	3.36
Ald 12	aldicarb 2001	3.36
Ald 13	aldicarb 2001	3.36
Ald 14	aldicarb 2001	3.36
Ald 15	aldicarb 2001	3.36
FOS 1	fosthiazate	3
FOS 2	fosthiazate 2001 oxamyl 1997 & 1993	3.3
FOS 3	fosthiazate 2001 oxamyl 1997 & 1993	fosthiazate- 3
FOS 4	1,3-dichloropropene 2001 fosthiazate 2001	 fosthiazate- 3
FOS 5	fosthiazate 2001 oxamyl 1996 & 1991 aldicarb 1995 & 1993	3 5.5 1993- 3.36, 1995- 0.51g /100m row
FOS 6	fosthiazate 2001 oxamyl 1996 & 1991 aldicarb 1995 & 1993	3 5.5 1993- 3.36, 1995- 0.51g /100m row
FOS 7	fosthiazate 2001 1,3-dichloropropene 2001 aldicarb 1996, 1991 & 1986	3 3.36
FOS 8	fosthiazate 2001 1,3-dichloropropene 2001	3
FOS 9	fosthiazate 2001 1,3-dichloropropene 2001	3
FOS 10	fosthiazate 2001 1,3-dichloropropene 2001	3

Table 5 continued: Nematicide application history for incubation study soils

Soil	Nematicide application history	Rate (kg ai/ha)
OX 2	oxamyl 2001	5.5
OX 3	oxamyl 2001	5.5
OX 4	oxamyl 2001 1,3-dichloropropene 2000	5.5
OX 5	oxamyl 2001 aldicarb 1995	5.5
OX 7	oxamyl 2001	5.5
OX 8	oxamyl every 4/5 years since 1979	5.5
OX 9	oxamyl 2001 & 2000	5.5
OX 10	oxamyl 2001 & 2000	5.5
OX 11	oxamyl 2001	5.5
OX 12	oxamyl 2001 aldicarb 1997 & 1993	5.5 3.36

Table 6: Estimated DT50 values (days) and corresponding r^2 values for the fitted curves with standard error of the mean (n=2) for soils that received three successive applications of oxamyl. [a]

Soil	1st application		2nd application		3rd application	
	DT50	R2	DT50	R2	DT50	R2
OX2	11.6 ^{A} (±2.4)	0.76 (±0.16)	2.7 ^{B*} (±0.5)	0.99 (±1.5^{-4})	1.5 ^{B*} (±0.0)	0.99 (±0.00)
OX3	2.1 ^{A} (±0.3)	0.99 (±3.25^{-3})	4.1 ^{A} (±1.1)	0.99 (±0.01)	4.7 ^{A} (±3.5)	0.57 (±0.13)
OX4	4.4 ^{A} (±1.1)	0.99 (±1.92^{-3})	1.2 ^{AB**} (±0.1)	0.99 (±0.00)	1.7 ^{AC} (±0.1)	0.99 (±5.65^{-5})
OX5	23.9 ^{A} (±5.4)	0.63 (±0.23)	9.9 ^{AB} (±4.3)	0.76 (±0.21)	2.6 ^{B*} (±0.2)	0.99 (±2.0^{-4})
OX7	7.4 ^{A} (±6.1)	0.82 (±0.09)	2.3 ^{AB*} (±0.2)	0.99 (±1.2^{-4})	1.6 ^{AC} (±0.0)	0.99 (±1.28^{-3})
OX8	7.3 ^{A} (±0.7)	0.99 (±1.35^{-3})	2.9 ^{B**} (±0.5)	0.99 (±2.14^{-3})	3.9 ^{B*} (±0.9)	0.99 (±8.33^{-3})
OX9	4.7 ^{A} (±0.2)	0.99 (±1.8^{-3})	1.8 ^{B**} (±0.2)	0.99 (±4.45^{-4})	1.5 ^{B**} (±0.1)	0.99 (±4.26^{-3})
OX10	1.1 (±0.1)	0.99 (±0.00)	ND		ND	
OX11	21.9 ^{A} (±6.9)	0.45 (±0.22)	14.9 ^{A} (±5.8^{-7})	0.74 (±0.16)	12.3 ^{A} (±5.4)	0.80 (±0.27)
OX12	2.3 ^{A} (±0.2)	0.99 (±2.35^{-4})	3.9 ^{AB} (±2.4)	0.99 (±2.85^{-4})	1.5 ^{B*} (±0.1)	0.99 (±3.62^{-3})

All values are the mean (n=2)
*[a] DT50 values followed by the same superscript capital letter in the row are not significantly different. Letters followed by * are significant at p<0.1 and letters followed by ** are significant at p<0.05.*
ND, Not determined

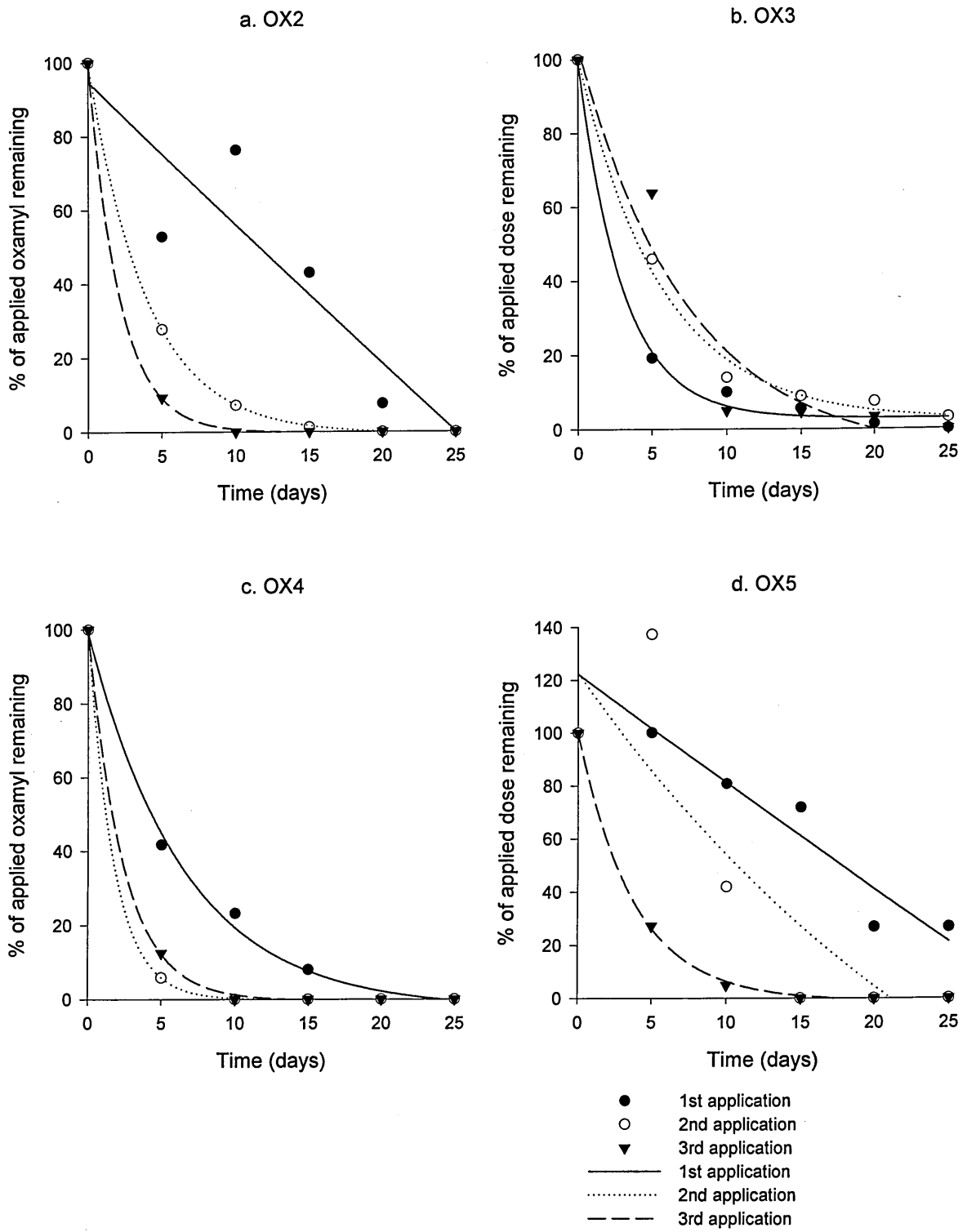


Figure 7: Degradation of oxamyl in soils OX 2,3,4 and 5 after three successive applications. Data points are the mean (n=2) presented as a percentage of the day 0 concentration.

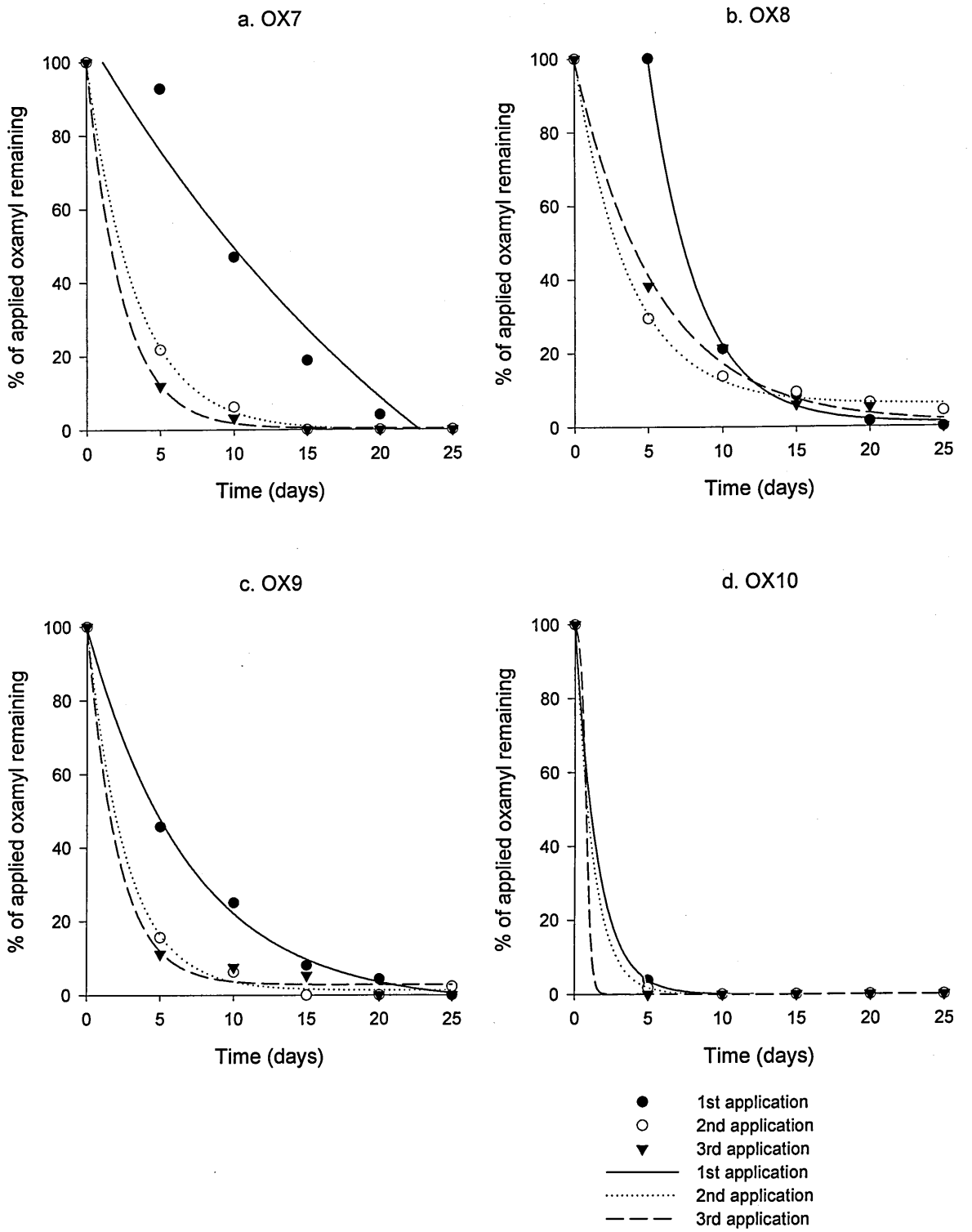


Figure 7 continued: Degradation of oxamyl in soils OX 7,8,9 and 10 after three successive applications. Data points are the mean (n=2) presented as a percentage of the day 0 concentration.

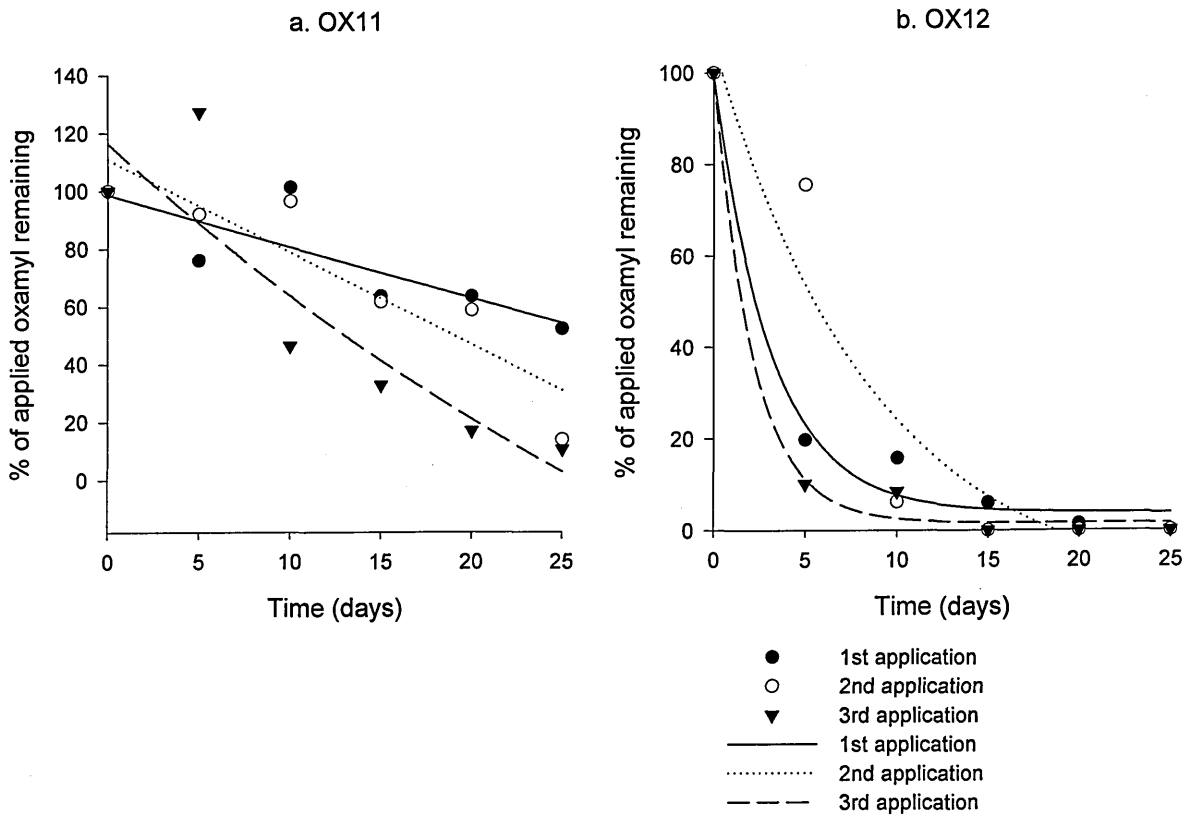


Figure 7 continued: Degradation of oxamyl in soils OX 11 and 12 after three successive applications. Data points are the mean (n=2) presented as a percentage of the day 0 concentration.

2.3.2 Aldicarb

As with oxamyl, problems arose when analysing the aldicarb degradation data from the incubation study. A high degree of variation occurred within samples and as such, in some cases, curves could not be fitted or DT50 values calculated. Similarly, statistically analysing the differences between the DT50 values of the three applications proved difficult because of the high degree of variability between replicates, and the low number of replicates (*n=2*), as can be seen from the standard error values in Table 7. As such, the 90% confidence limit was considered significant. The DT50 values that could be calculated and their corresponding r^2 values, and any significant differences between applications are displayed in Table 7. The oxidation products, aldicarb sulphoxide and aldicarb sulphone, contribute to the nematicidal activity of aldicarb within the soil and as

such the DT50 values are calculated from the degradation curves of the total toxic residues of all three chemicals combined (TTR).

The degradation patterns for parent aldicarb, aldicarb sulphoxide, aldicarb sulphone and the concentrations of all three residues combined are displayed in Figure 8. From the DT50 values, statistical analysis and degradation patterns, it is possible to split the soils into three groups: One, those soils in which degradation proceeded faster in the second and third applications than in the first; two, soils in which all three applications were degraded rapidly; and three, soils in which degradation was slow in all three applications. Six of the 15 soils, Ald 4, 6, 7, 10, 14 and 15, fall into the first category and of these the second and third applications were degraded significantly faster ($P< 0.1$ and 0.05) than the first in two soils, Ald 4 and Ald 15. The second and third applications applied to soil Ald 7 were degraded faster than the first, with DT50 values of 2.8, 1.5 and 10.8 days respectively. However, the difference is not significant, probably because of the high degree of variation between the two first application replicates. Similarly, the DT50 values for the second and third applications applied to soil Ald 14 (3.8 and 3.4 days respectively) are a lot less than that for the first application, 12.1 days, and again there is no significant difference. As with soil Ald 7, variation between the two first application replicates is most likely the cause of the lack of significant difference between applications. From Figure 8 it is possible to see the large difference in the rate of degradation of the total toxic residues between the first application and the subsequent applications for soils in this category. Figure 8 also shows the degradation patterns of each of the three individual aldicarb residues. For all soils in this category, parent aldicarb is quickly degraded in all three applications.

Table 7: Estimated DT50 values (days) for aldicarb TTR and corresponding r^2 values for the fitted curves with standard error of the mean (n=2) for soils that received three successive aldicarb applications. [a]

Soil	1st application		2nd application		3rd application	
	DT50	R2	DT50	R2	DT50	R2
Ald1	ND		ND		29.1 (±0.6)	0.99 ($\pm 7.0^{-4}$)
Ald2	ND		24.5 (±19.3)	0.56 (±0.34)	ND	
Ald3	11.6 ^{A} (±4.7)	0.86 (±0.14)	8.8 ^{A} (±1.8)	0.90 (±0.03)	12.3 ^{A} (±0.7)	0.88 (±0.07)
Ald4	3.2 ^{A} (±0.0)	0.99 ($\pm 3.9^{-3}$)	1.6 ^{B**} (±0.1)	0.99 ($\pm 2.13^{-3}$)	1.0 ^{C**} (±0.1)	0.99 ($\pm 4.5^{-5}$)
Ald5	ND		ND		ND	
Ald6	ND		6.8 ^{A} (±3.4)	0.91 (±0.08)	1.1 ^{A} (±0.2)	0.98 (±0.02)
Ald7	10.8 ^{A} (±5.2)	0.94 (±0.06)	2.8 ^{A} (±0.6)	0.98 (±0.02)	1.5 ^{A} (±0.2)	0.98 (±0.02)
Ald8	11.8 ^{A} (±5.1)	0.54 (±0.08)	29.5 $^{B}{}_{*}{}^{**}$ (±0.4)	0.84 (±0.07)	14.6 ^{A} (±1.0)	0.97 ($\pm 8.9^{-3}$)
Ald9	2.4 ^{A} (±0.3)	0.73 (±0.25)	3.9 ^{A} (±0.4)	0.99 ($\pm 1.7^{-4}$)	ND	
Ald10	4.3 ^{A} (±1.0)	0.99 ($\pm 1.48^{-3}$)	2.8 ^{A} (±0.3)	0.99 ($\pm 1.6^{-3}$)	ND	
Ald11	1.6 ^{A} (±0.6)	0.99 ($\pm 2.14^{-3}$)	2.3 ^{A} (±0.7)	0.99 ($\pm 1.13^{-3}$)	24.8 ^{A} (±22.9)	0.67 (±0.32)
Ald12	ND		7.1 ^{A} (±2.5)	0.93 ($\pm 7.16^{-3}$)	2.1 ^{A} (±0.5)	0.99 (±0.01)
Ald13	6.0 ^{A} (±4.6)	0.93 (±0.07)	ND		1.1 ^{A} (±0.1)	0.99 (±0.01)
Ald14	12.1 ^{A} (±3.3)	0.85 (±0.09)	3.8 ^{A} (±0.0)	0.97 (±0.01)	3.4 ^{A} (±1.9)	0.96 (±0.04)
Ald15	10.4 ^{A} (±2.1)	0.96 ($\pm 4.95^{-4}$)	2.7 ^{B*} (±0.1)	0.99 ($\pm 9.0^{-5}$)	1.5 ^{C*} (±0.2)	0.99 ($\pm 6.59^{-3}$)

All values are the mean (n=2)

[a] *DT50 values followed by the same superscript capital letter in the row are not significantly different. Letters followed by * are significantly different at P<0.1 and letters followed by ** are significantly different at P<0.05.*

ND, Not determined

This is not the case for aldicarb sulphoxide however, as aldicarb sulphoxide persists for longer in the first application than in the subsequent two applications for all six soils. The amount of aldicarb sulphone produced is small in all soils and previous application appears to have no effect on its persistence as there is little difference between applications. All six soils in this category had received one previous aldicarb application in the field (Table 5). In addition to this, Ald 4 had also received two oxamyl applications in the past.

Interestingly, soil Ald 6 had been fumigated with 1,3-dichloropropene prior to aldicarb application in the field but still demonstrated an increase in the rate of aldicarb degradation with successive application in the laboratory. The pH of these soils ranged from 6.3 in soil Ald 7 to 7.6 in Ald 6 and soils were classed as either sandy loam or clay loam (Table 4).

Three soils (Ald 9, 12 and 13) fall into category two because the first application as well as the second and third appeared to be degraded rapidly. Although no significant differences were calculated between applications for any of the three soils, those DT50 values that could be calculated show relatively rapid degradation. For example, the DT50 values for the first and second applications applied to soil Ald 9 were 2.4 and 3.9 days respectively. The longest DT50 was for the second application to soil Ald 12 and this was only 7.1 days. As with soils in the first category, parent aldicarb was degraded rapidly for all three applications applied to these three soils. However, less of a difference in the degradation of aldicarb sulphoxide between the three applications is evident in these soils, although the third application does appear to be degraded faster than the first in all three soils. The concentration of aldicarb sulphone residues is again small and as with category one soils there appears to be little difference between the three applications. The pH of these soils ranged from 5.8 to 6.7 and all three are sandy loam soils.

The degradation patterns and the DT50 values that could be calculated for the six soils in category three show much slower degradation of total toxic aldicarb residues and no increase in the rate of degradation with subsequent aldicarb application. Soils Ald 1, 2, 3, 5, 8 and 11 make up category three, although soil Ald 11 could possibly be classed as category two were it not for the comparatively high, but variable (standard error of ±22.9 days), third application DT50 of 24.8 days. The concentration of parent aldicarb residues declined slowly in Ald 2, 3 and 5 and the concentration of aldicarb sulphoxide residues was much higher and more persistent in all soils in this category than in categories one and

two. In soils Ald 2 and 5, aldicarb sulphoxide was continuing to accumulate in all three applications at day 30. The concentration of aldicarb sulphone did, however, vary between the six soils. Higher concentrations were seen in soils Ald 1 and Ald 8, whereas lower concentrations were seen in the remaining four soils. Aldicarb sulphone residues were still accumulating by day 30 in all three applications applied to Ald 1. There appears to be little difference between the three successive applications in any of the soils except Ald 3 and Ald 8 in which increased concentrations of aldicarb sulphoxide were seen with the second and third applications, resulting in the higher total toxic residues observed with these doses. Three of the soils, Ald 2, 3 and 5, had high organic matter contents of 70, 52 and 36% respectively. Also, the pH range of soils in this category is slightly lower than that of the other two categories, ranging from 5.5 in soil Ald 5 to 6.7 in Ald 1 and 7.3 in Ald 11.

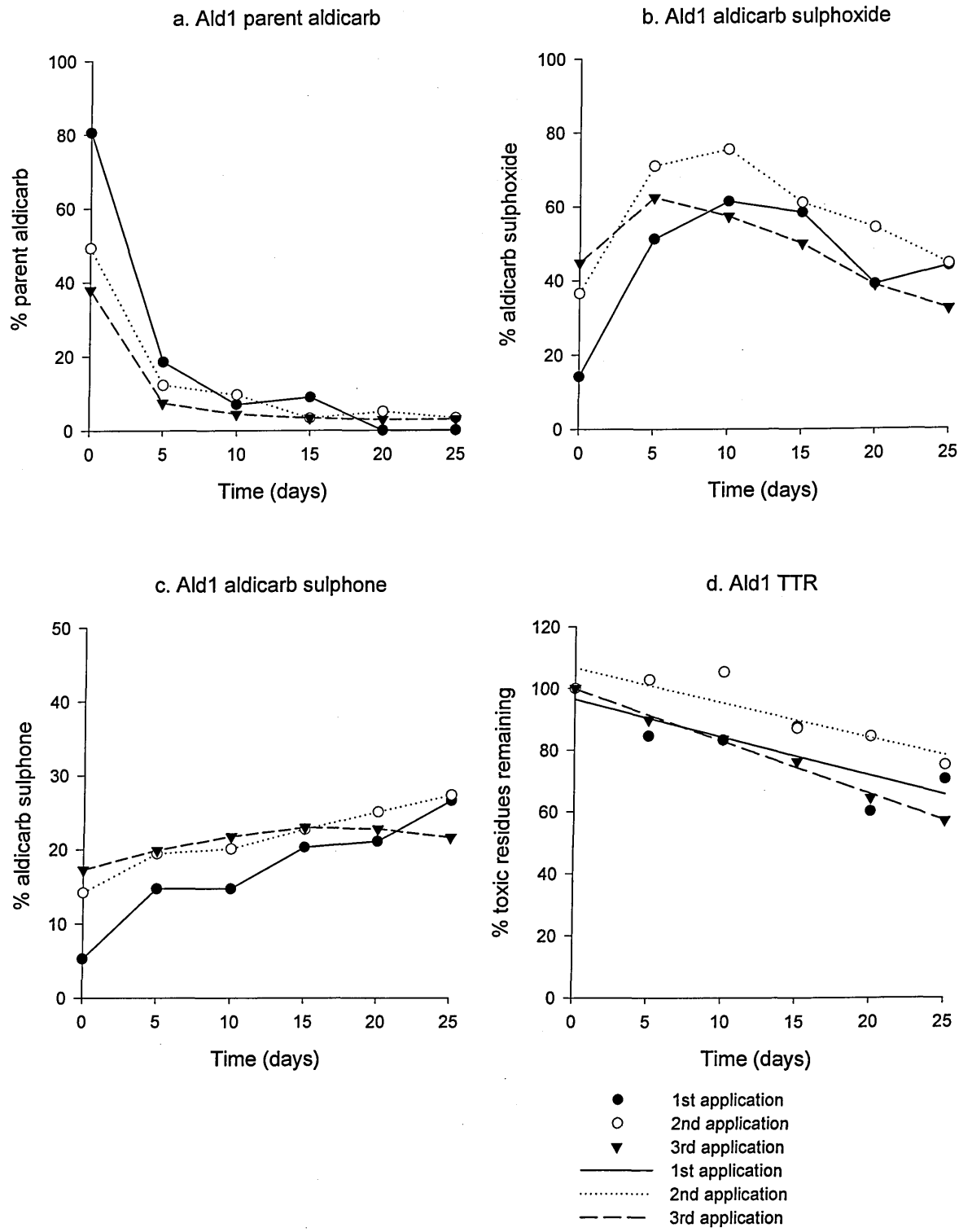


Figure 8: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 1 after three successive applications in the laboratory.

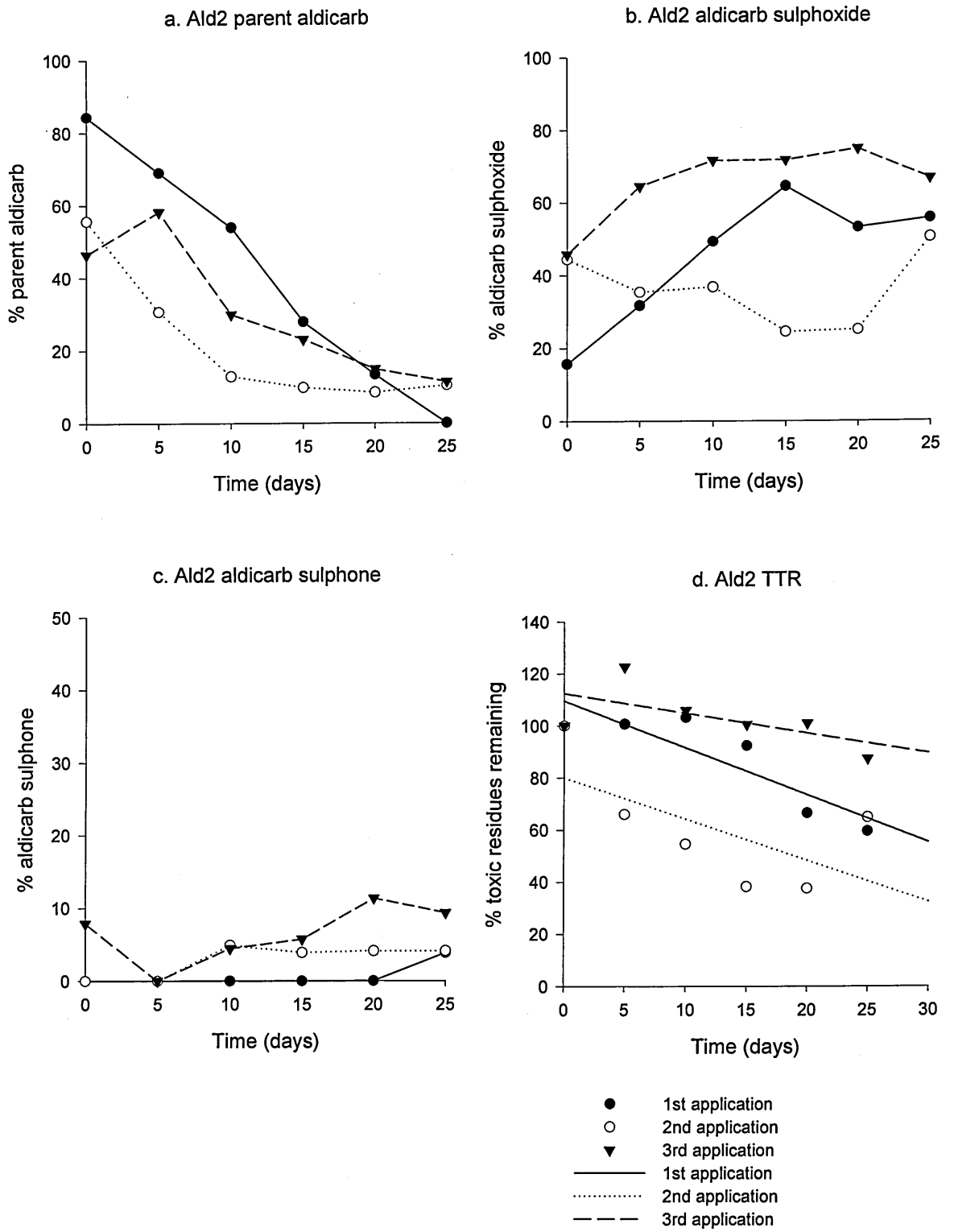


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 2 after three successive applications in the laboratory.

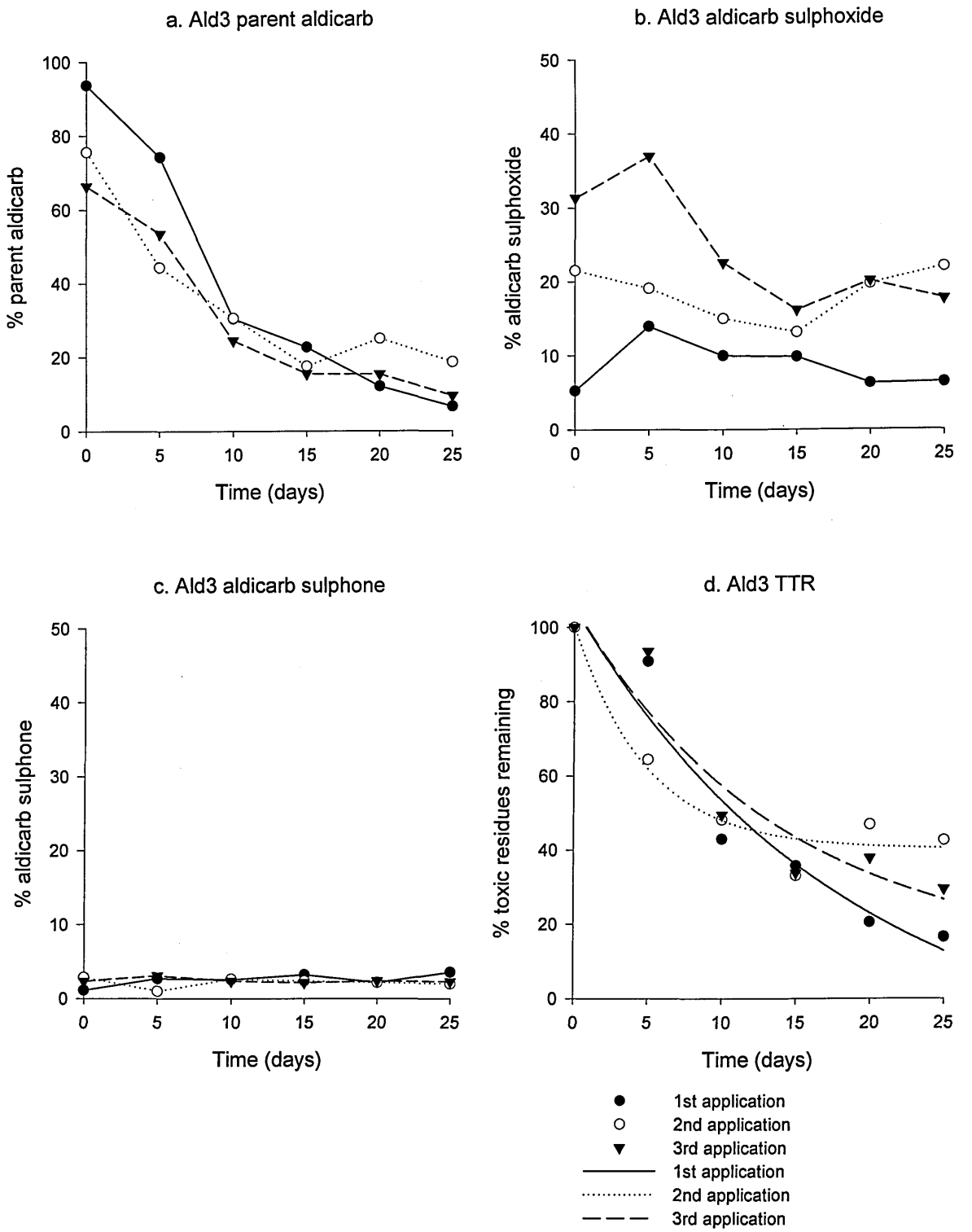


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 3 after three successive applications in the laboratory.

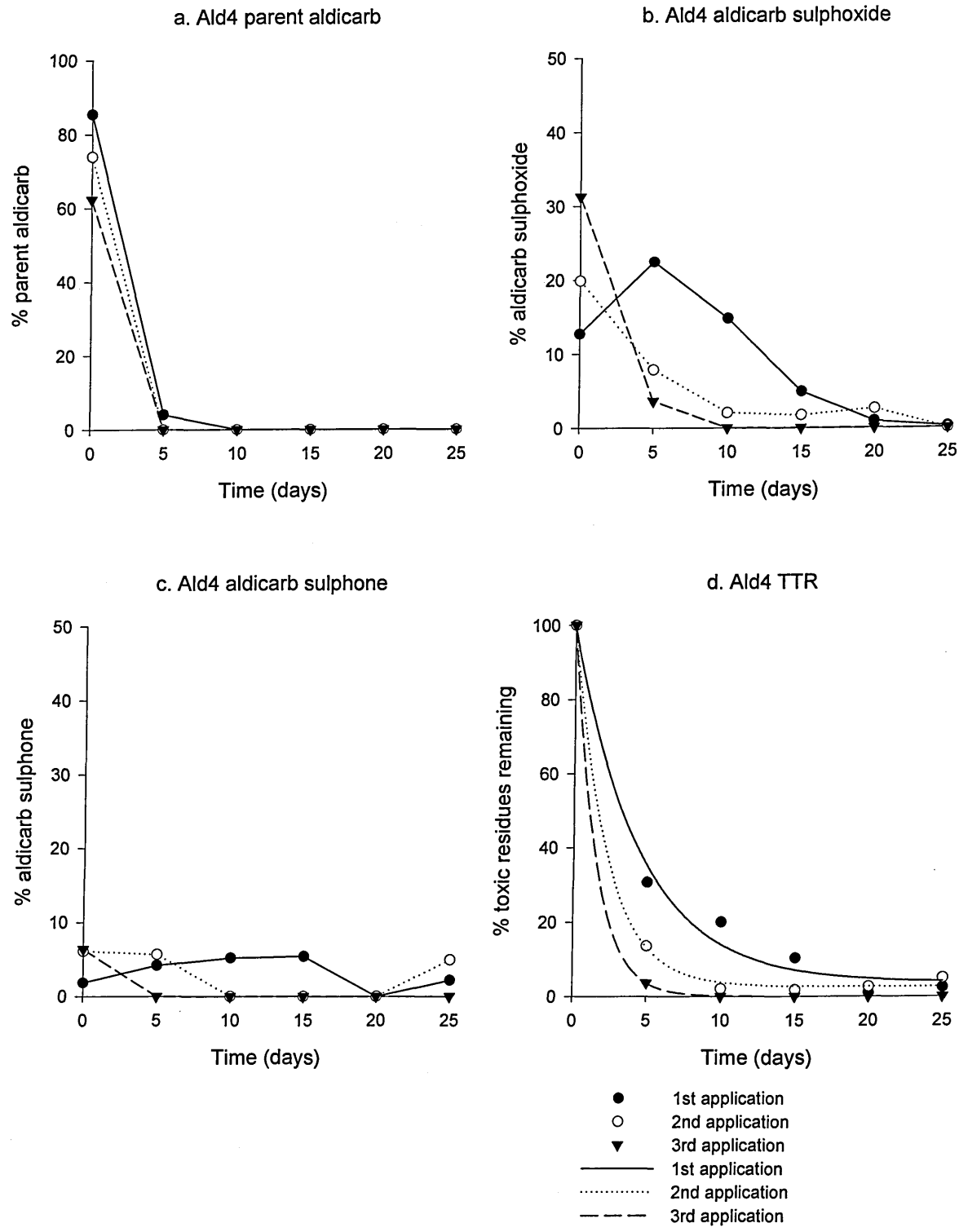


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 4 after three successive applications in the laboratory.

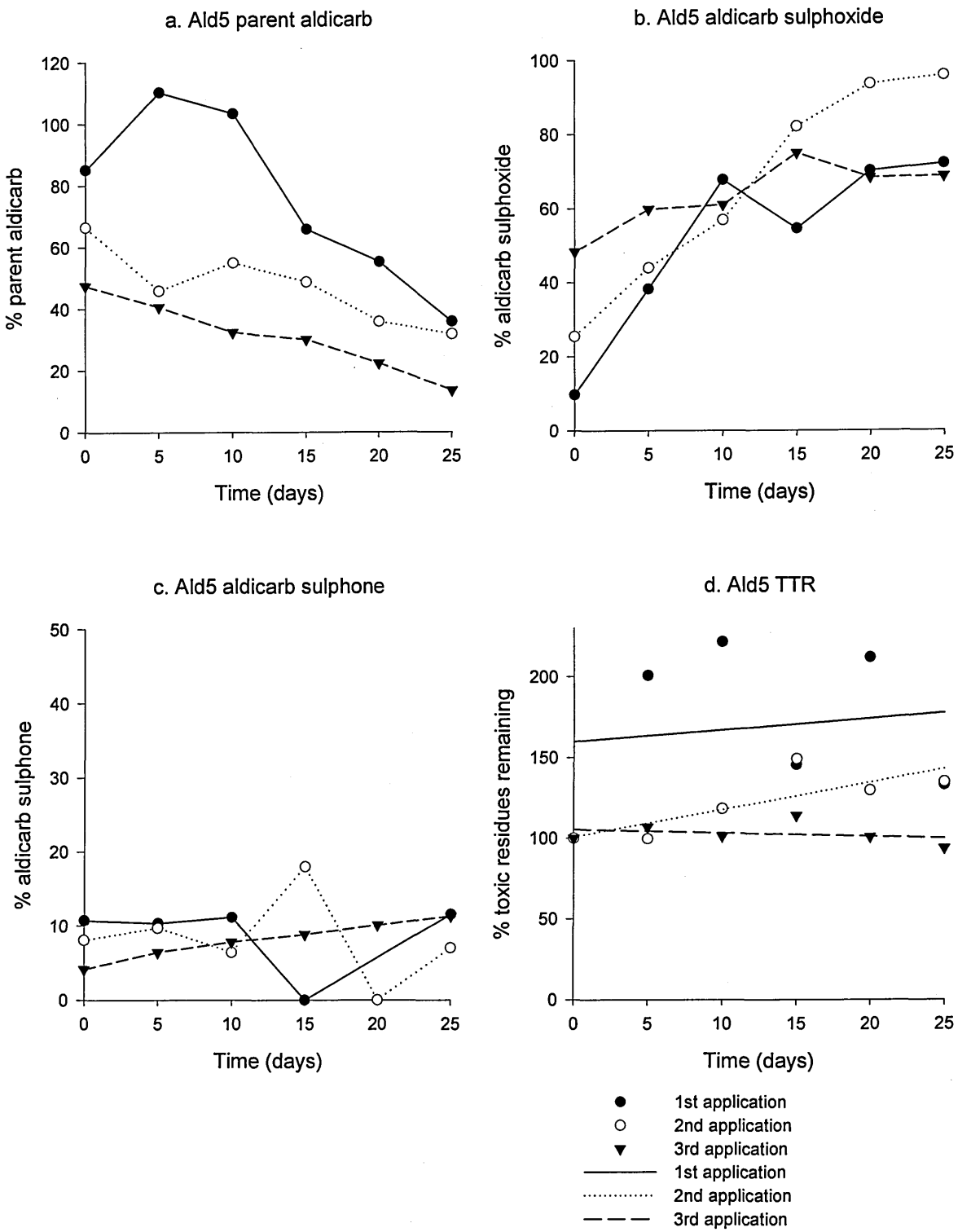


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 5 after three successive applications in the laboratory.

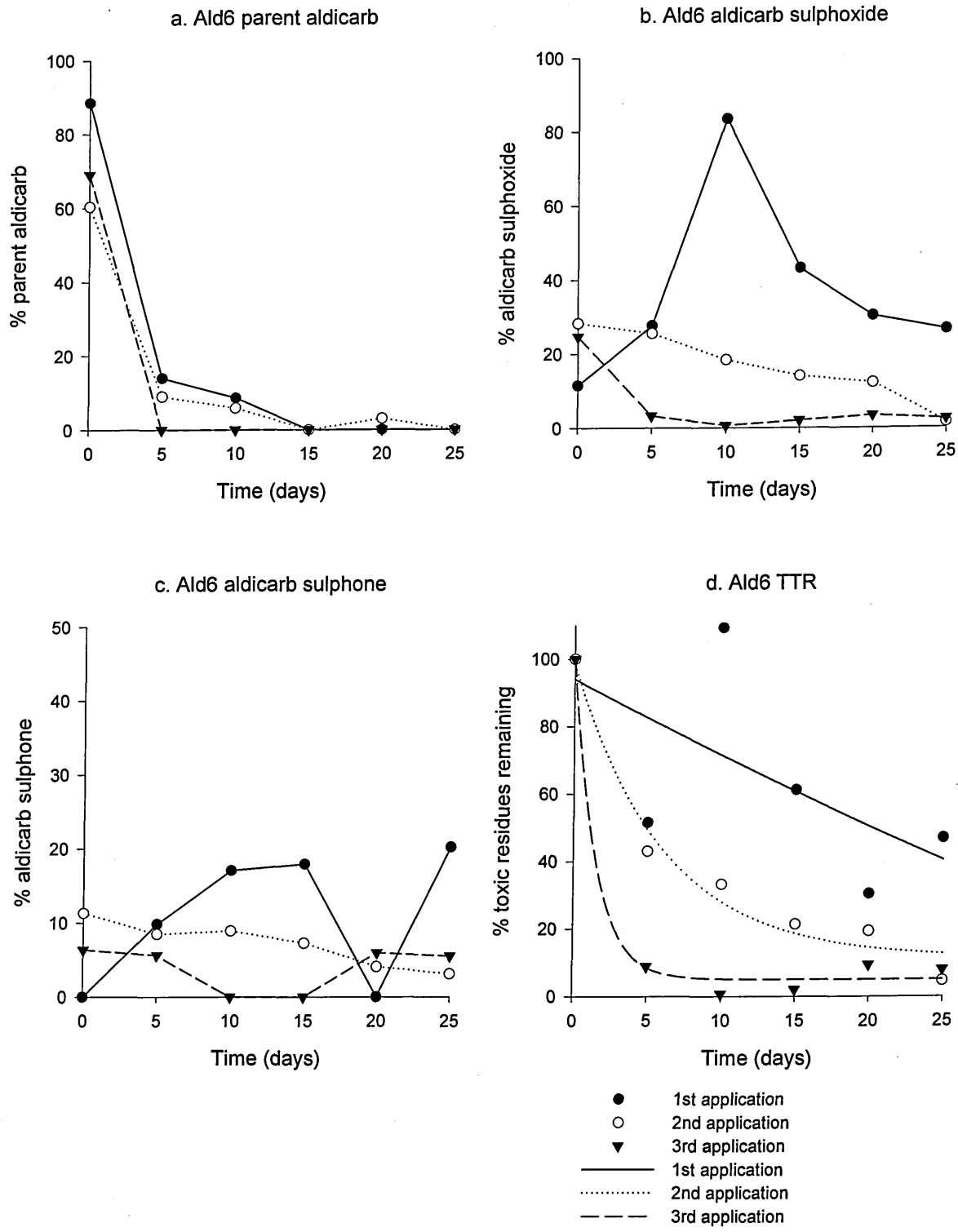


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 6 after three successive applications in the laboratory.

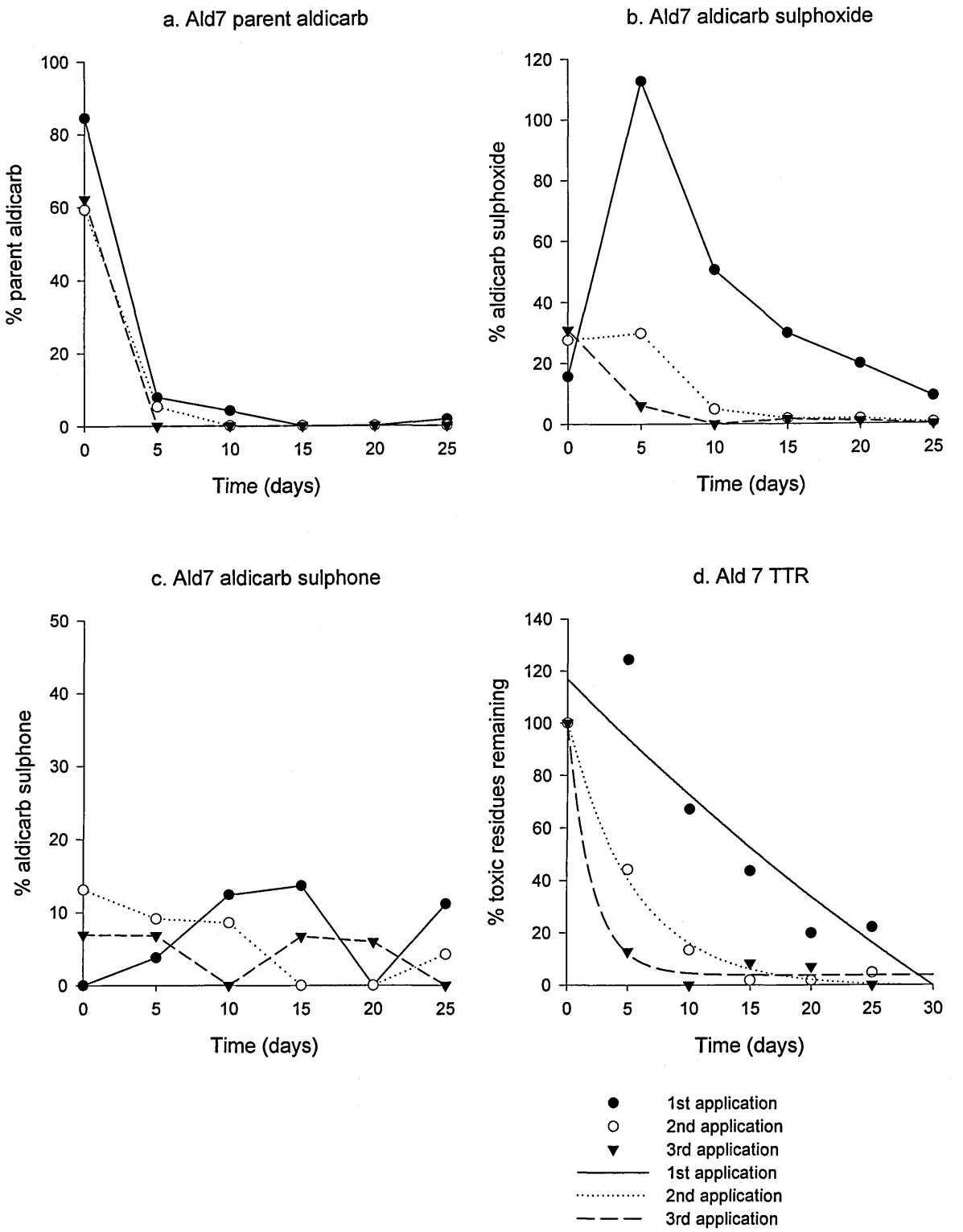


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 7 after three successive applications in the laboratory.

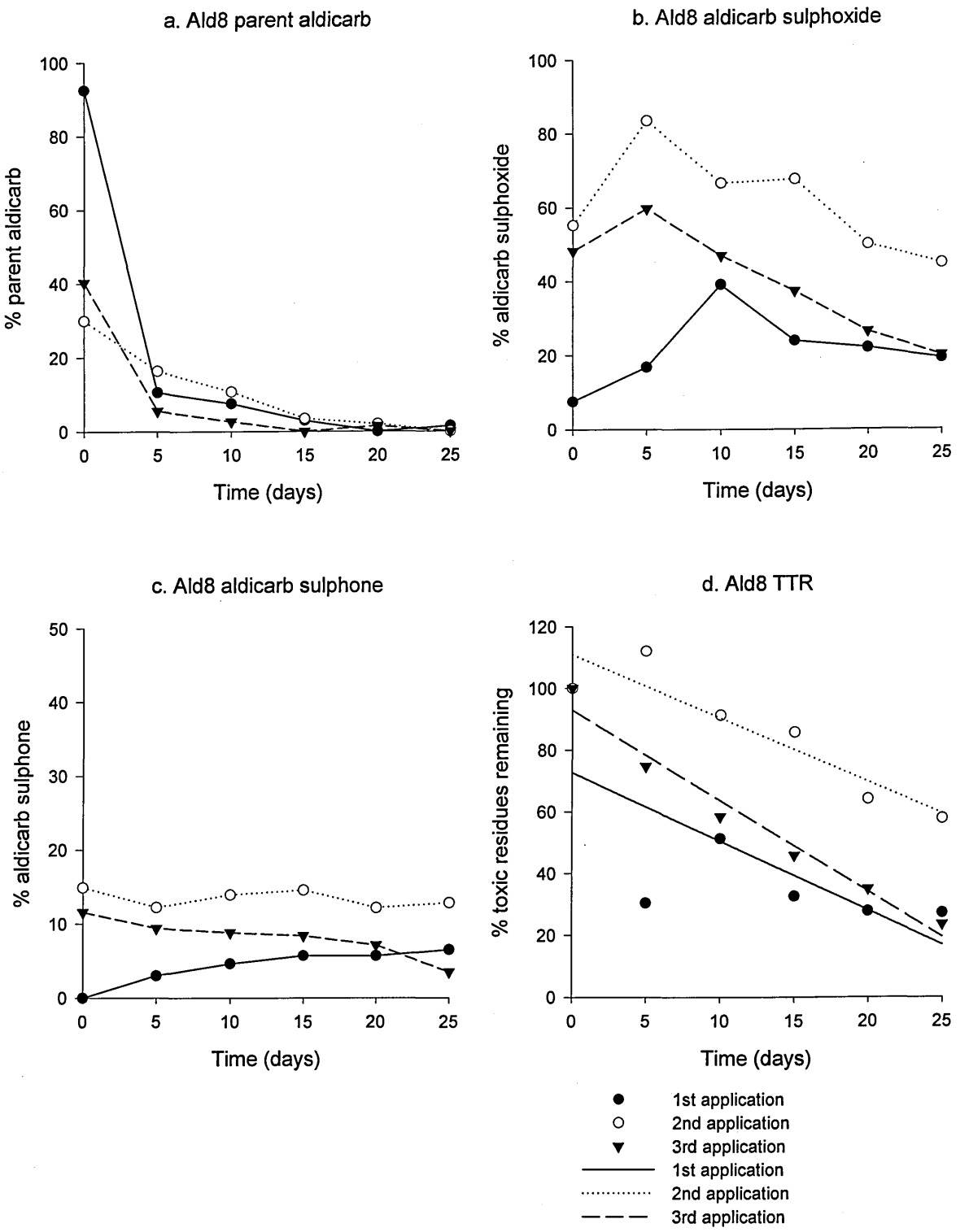


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 8 after three successive applications in the laboratory.

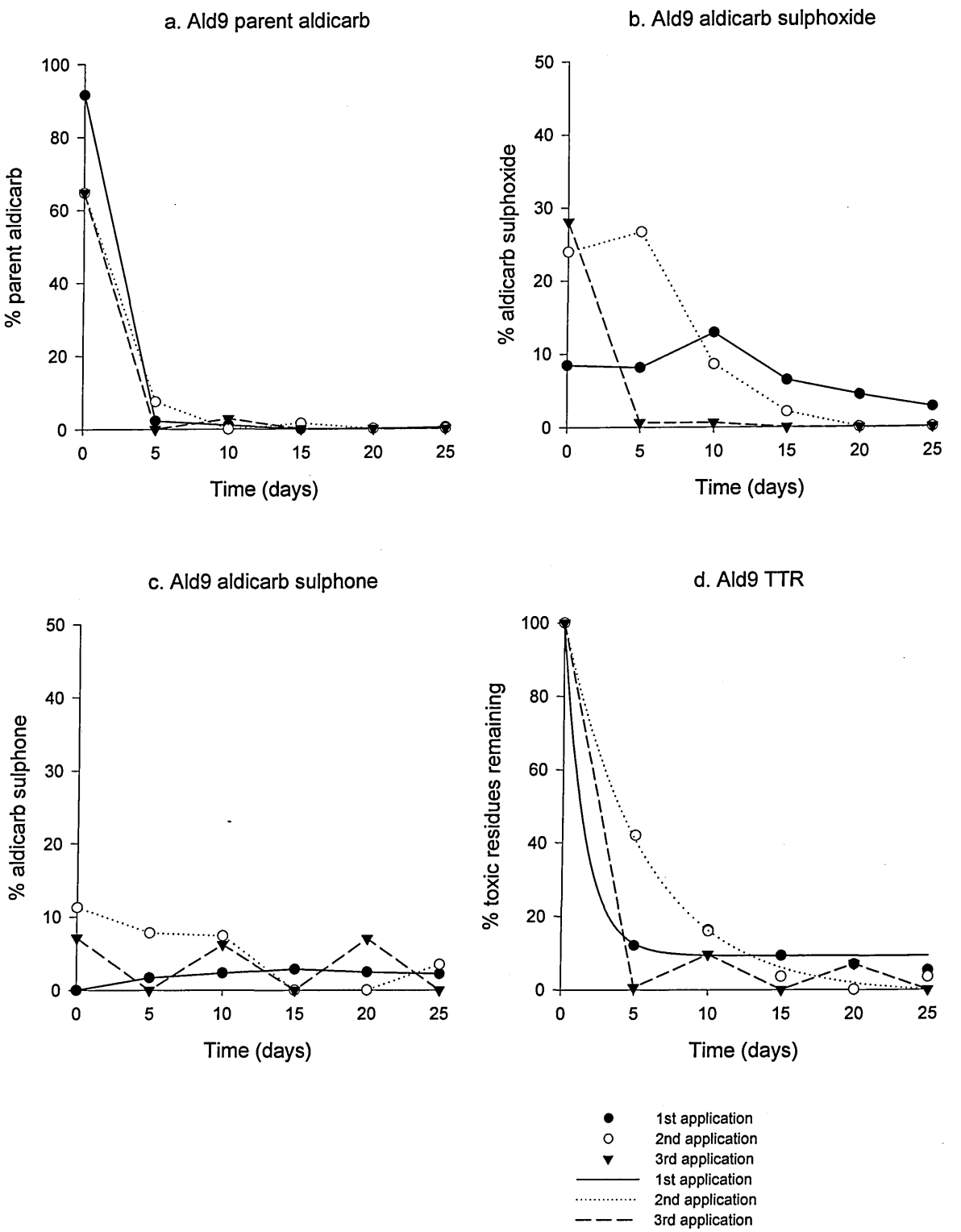


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 9 after three successive applications in the laboratory.

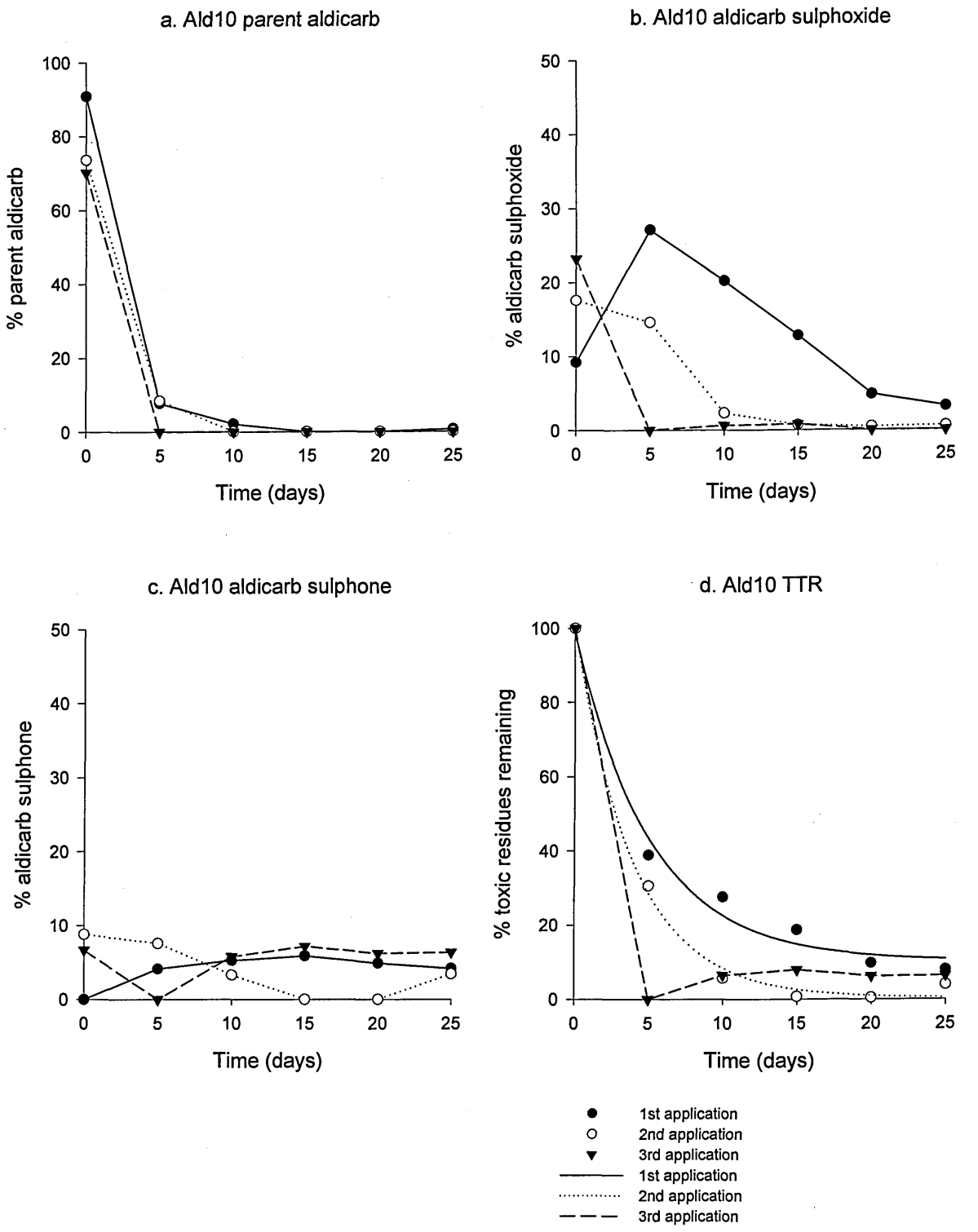


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 10 after three successive applications in the laboratory.

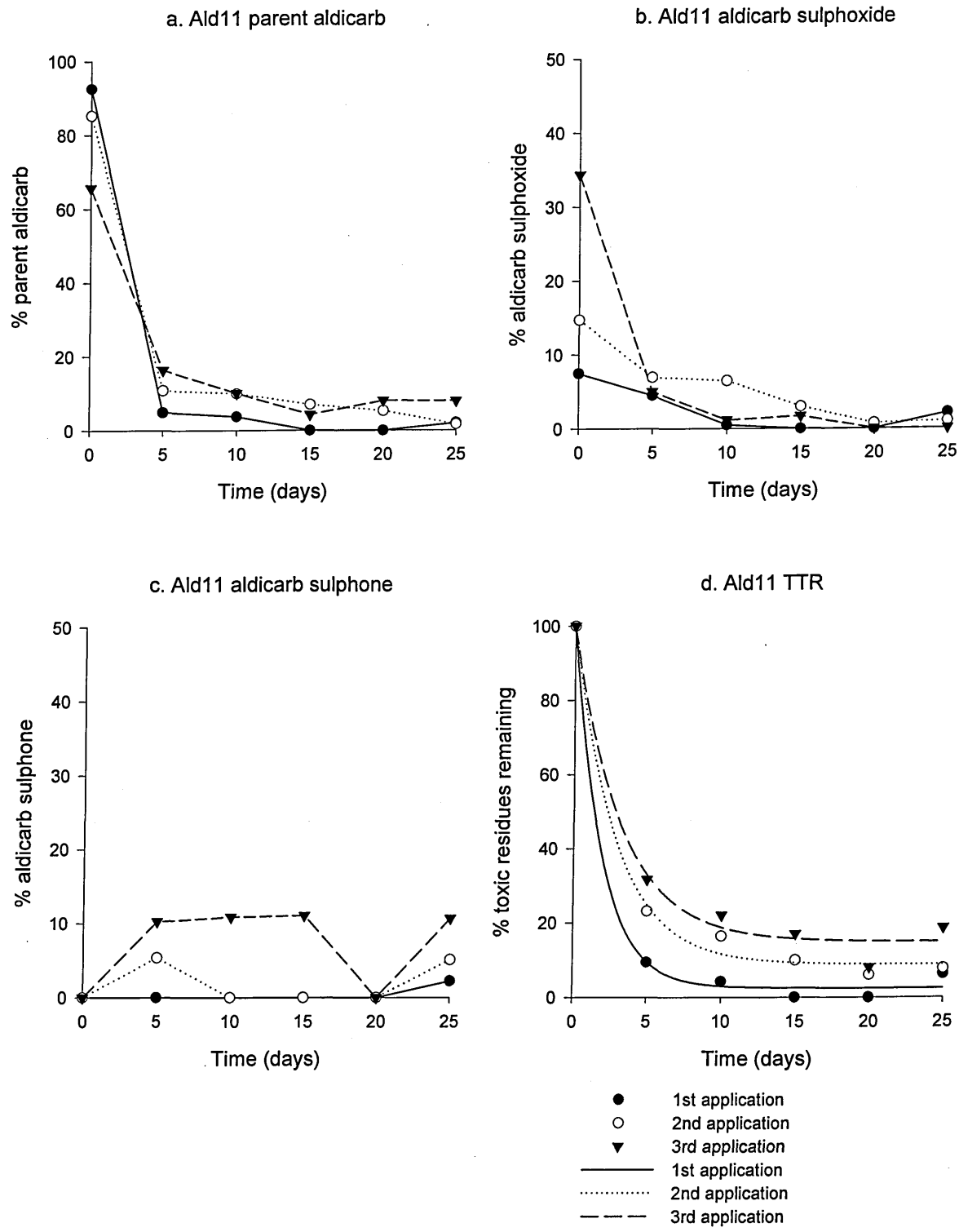


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 11 after three successive applications in the laboratory.

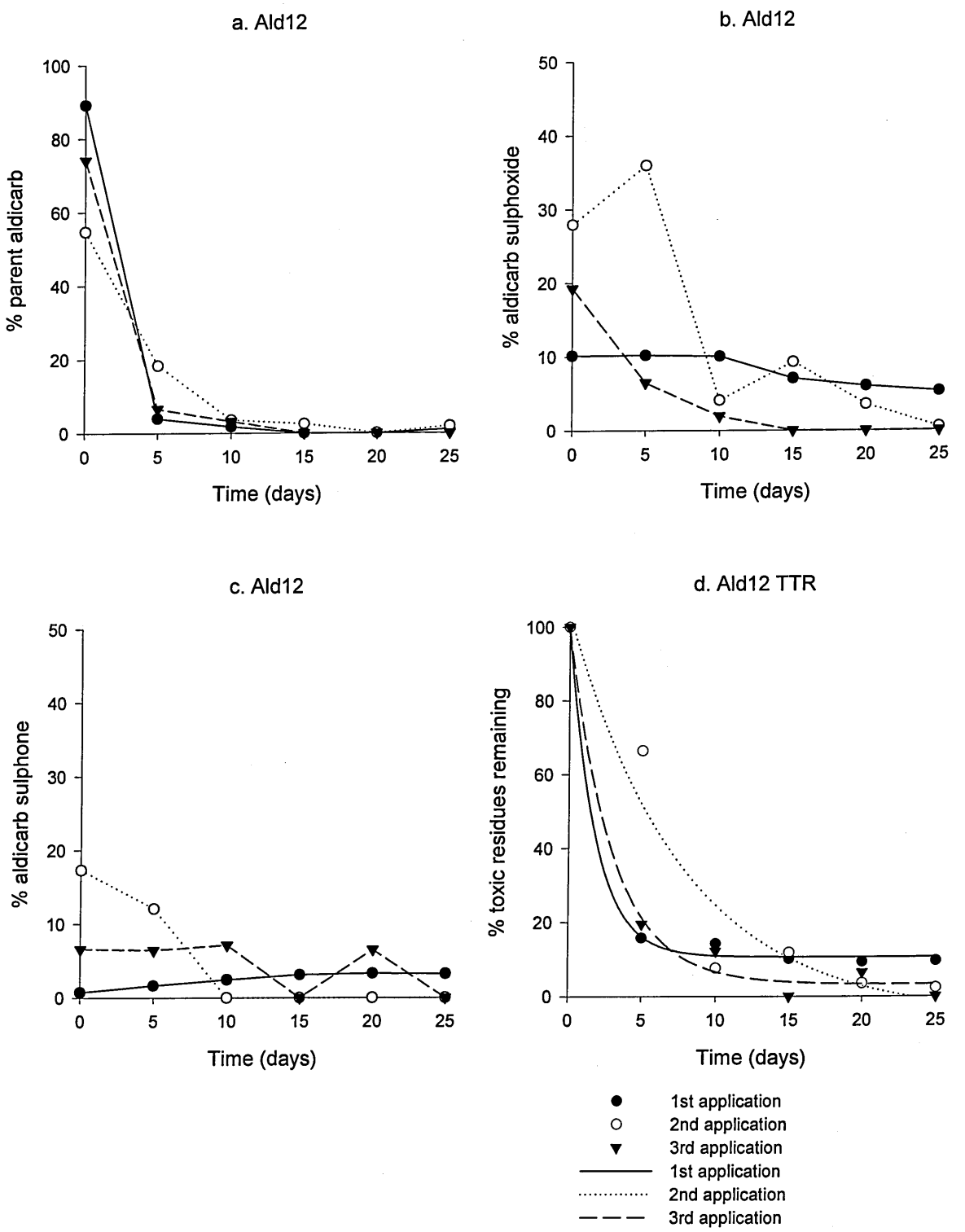


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 12 after three successive applications in the laboratory.

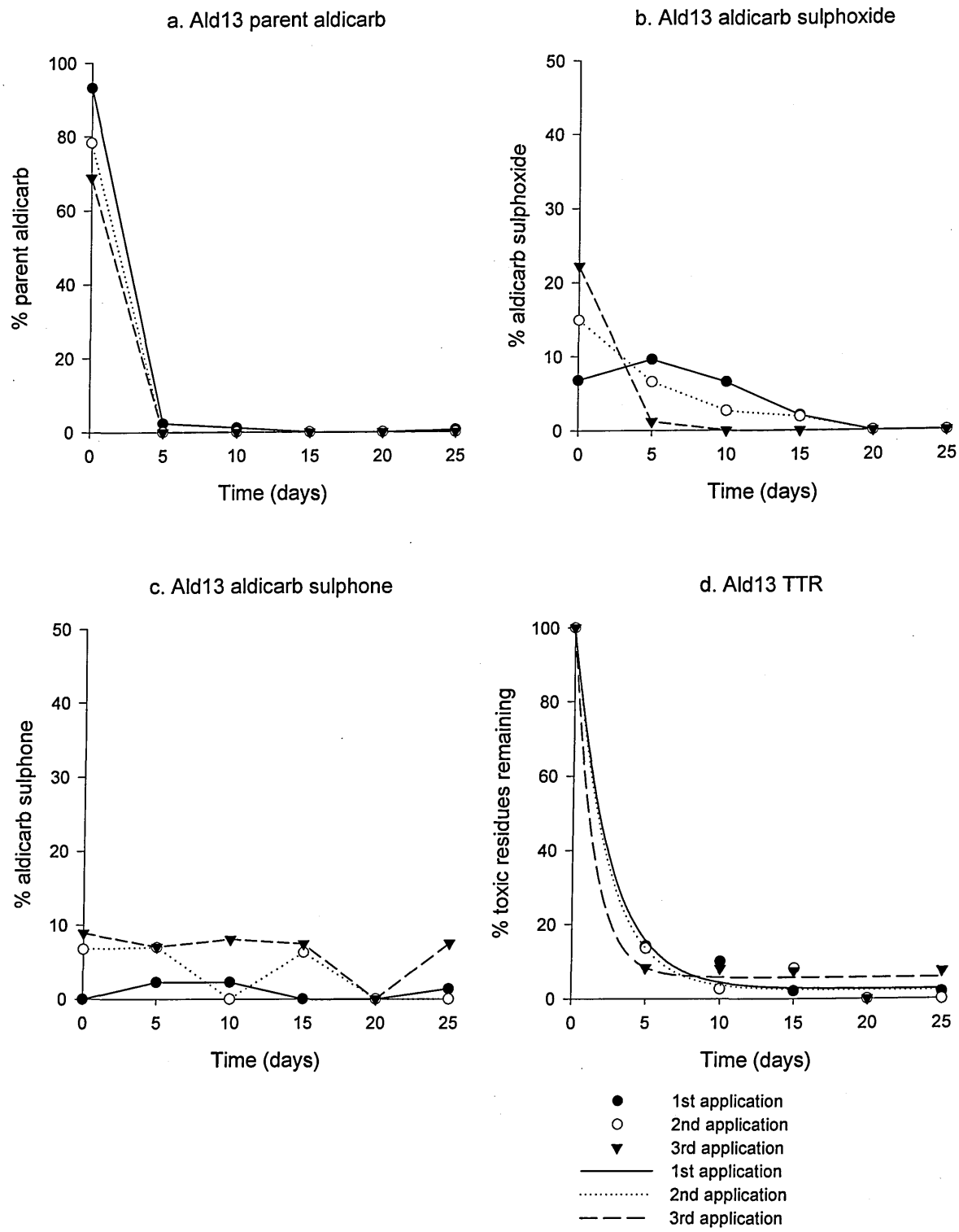


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 13 after three successive applications in the laboratory.

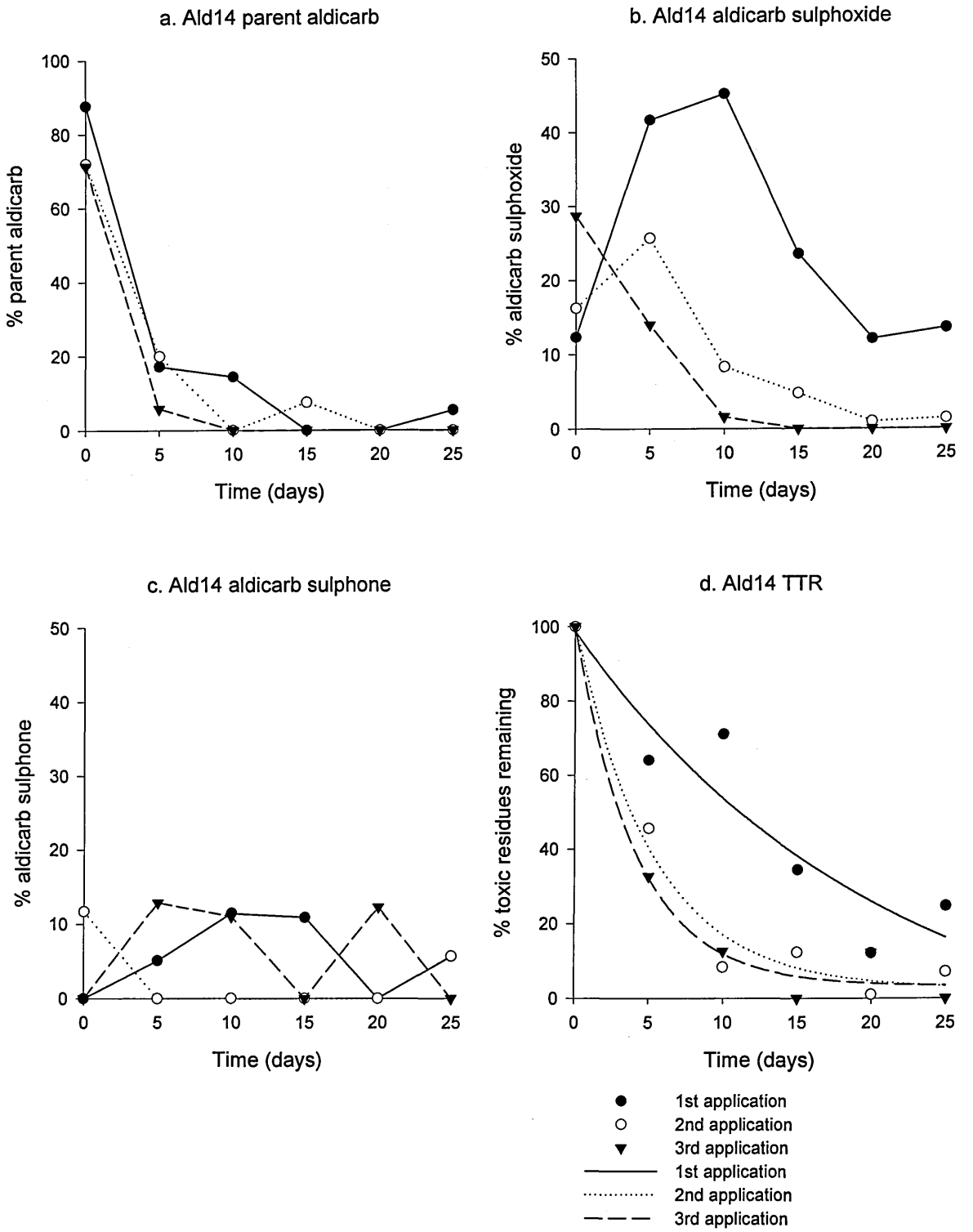


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 14 after three successive applications in the laboratory.

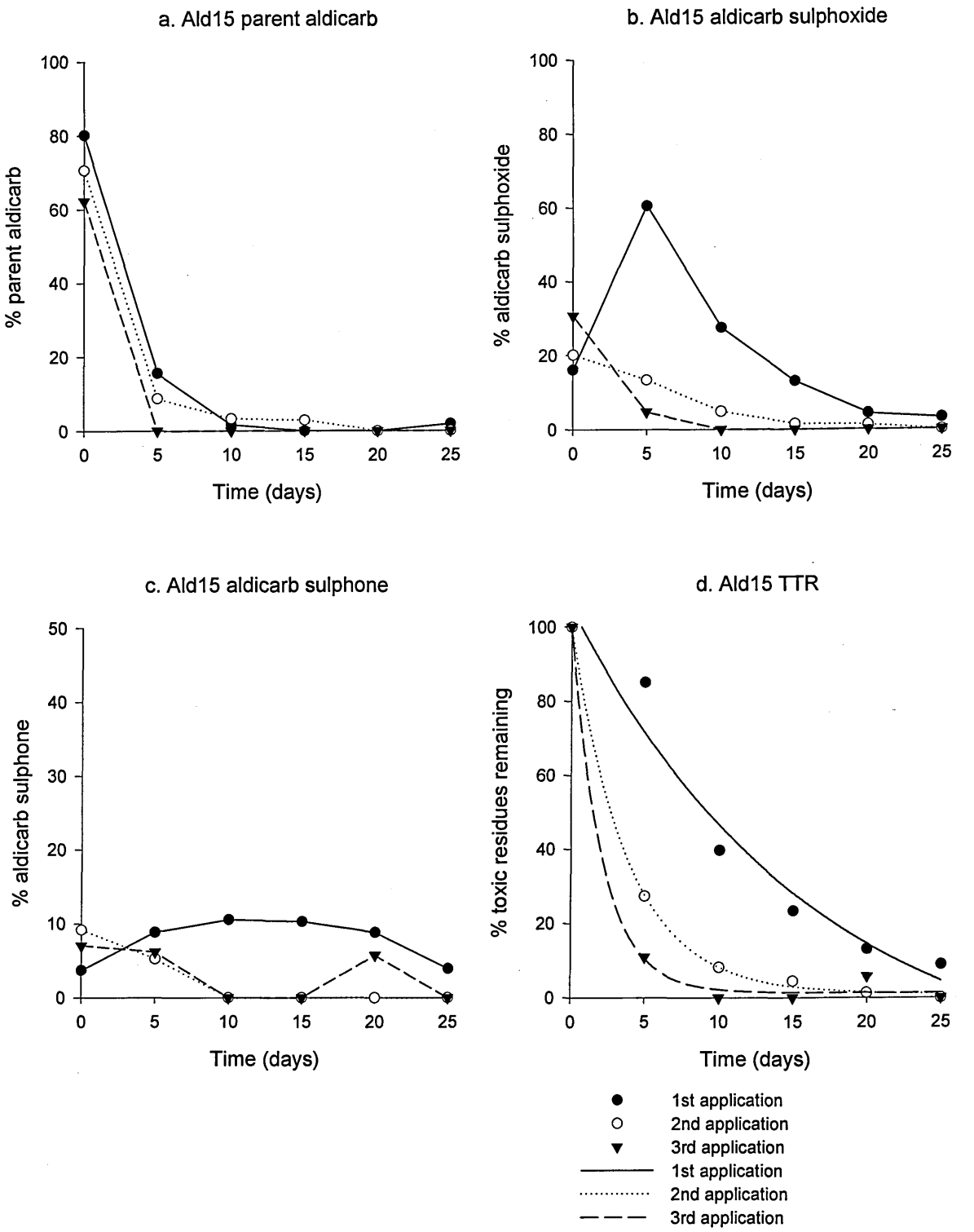


Figure 8 continued: Degradation of parent aldicarb (a), aldicarb sulphoxide (b), aldicarb sulphone (c) and the combined total toxic residues (d) in soil Ald 15 after three successive applications in the laboratory.

2.3.3 Fosthiazate

Fosthiazate degradation patterns after three successive applications are displayed in Figure 9. Due to the erratic nature of the data, points are displayed with a fitted curve to best show the degradation trend. The time taken to degrade 50% of each of the three fosthiazate applications (DT50) and the corresponding r^2 values are displayed in Table 8. As with the aldicarb and oxamyl degradation, it was difficult to fit curves to the fosthiazate data. For a number of the soils, the percentage of variance accounted for by the fitted curve was too low (*e.g.,* less than 45%) to warrant using it to calculate the DT50 value. Of those for which DT50 values were calculated, the r^2 values are still low in many cases. Also, there is a large degree of variation between replicates as shown by the standard error values. Due to the large standard errors, low number of replicates ($n=2$), large number of missing DT50 values and lack of enhanced degradation in the second and third applications as seen from Figure 9, differences between applications were not statistically analysed.

The most striking feature for all of the fosthiazate-treated soils is the slower degradation in the second and third applications than the first. The concentration of fosthiazate within the soil samples is actually accumulating as subsequent applications are applied to soil samples within which the previous application has not yet been fully degraded. The DT50 values for fosthiazate were higher than those recorded for oxamyl and aldicarb degradation, with DT50 values ranging from 11.1 to 73.0 days. Fosthiazate might have persisted longer than this in those soils for which DT50 values could not be calculated. Five of the ten fosthiazate soils, FOS1, 7, 8, 9 and 10, had high organic matter contents of 20% or above (Table 4). In addition, soils FOS7, 8, 9 and 10, and also FOS4 had been fumigated with 1,3-dichloropropene prior to fosthiazate application in the field (Table 5). However, slow degradation was also recorded for soils FOS2, 3, 5 and 6 that had a comparatively low organic matter content and had not been fumigated. The pH range of the fosthiazate soils was similar to those treated with oxamyl and aldicarb, ranging from 5.9 to 7.6.

Table 8: Estimated DT50 values (days) and corresponding r^2 values for the fitted curves with standard error of the mean (n=2) for soils that received three successive fosthiazate applications.

Soil	1st application		2nd application		3rd application	
	DT50	R2	DT50	R2	DT50	R2
FOS1	ND		ND		ND	
FOS2	17.8 (±10.6)	0.80 (±0.06)	ND		61.7 (±23.4)	0.72 (±0.13)
FOS3	19.8 (±1.1)	0.89 (±0.08)	17.0 (±0.1)	0.96 (±0.03)	16.2 (±0.5)	0.97 ($\pm 9.25^{-3}$)
FOS4	19.7 (±7.5)	0.46 (±0.07)	13.8 (±4.5)	0.78 (±0.12)	47.6 (±4.2)	0.78 (±0.13)
FOS5	ND		31.0 (±6.2)	0.66 (±0.24)	11.1 (±2.9)	0.90 (±0.07)
FOS6	ND		20.0 (±6.8)	0.57 (±0.03)	19.7 (±12.4)	0.72 (±0.03)
FOS7	ND		ND		58.4 (±5.4)	0.61 (±0.15)
FOS8	ND		ND		73.0 (±2.4)	0.82 (±0.06)
FOS9	ND		37.0 (±12.5)	0.48 ($\pm 9.62^{-3}$)	51.0 (±1.7)	0.78 (±0.13)
FOS10	ND		ND		ND	

All values are the mean (n=2)
ND, Not determined

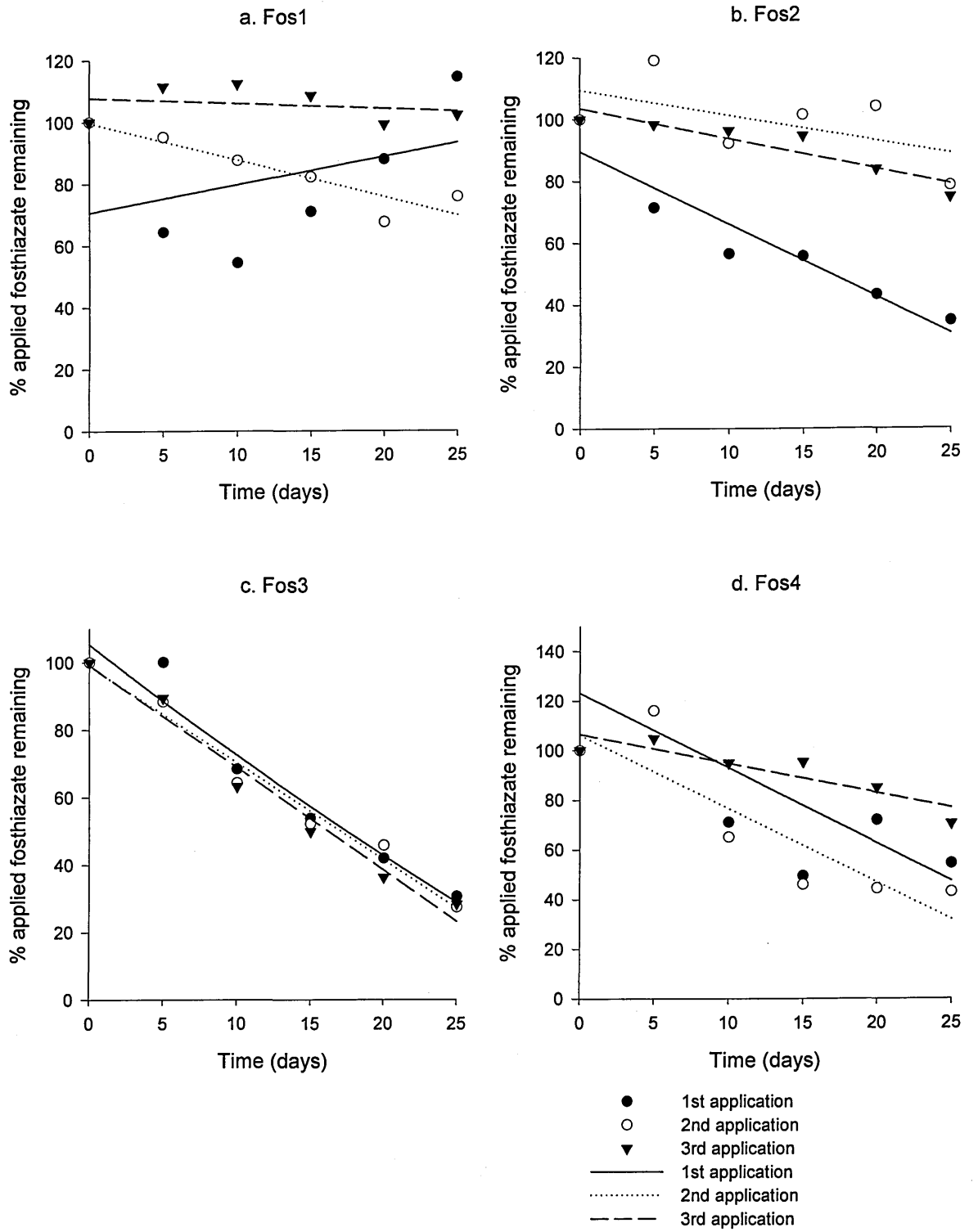


Figure 9: Degradation of fosthiazate in soils FOS 1, 2, 3 and 4 after three successive applications. Data points are the mean (n=2) presented as a percentage of the day 0 concentration.

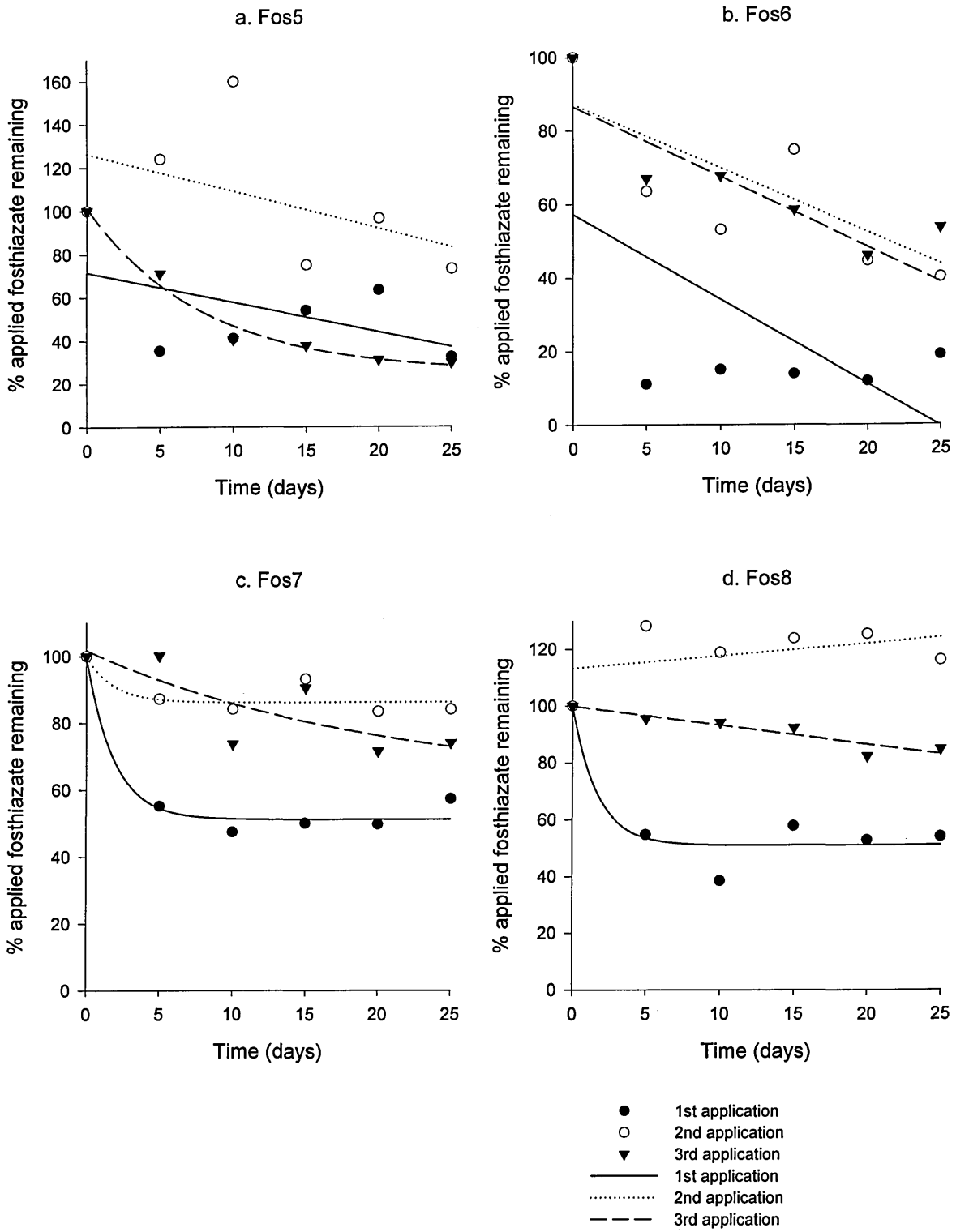


Figure 9 continued: Degradation of fosthiazate in soils FOS 5, 6, 7 and 8 after three successive applications. Data points are the mean (n=2) presented as a percentage of the day 0 concentration.

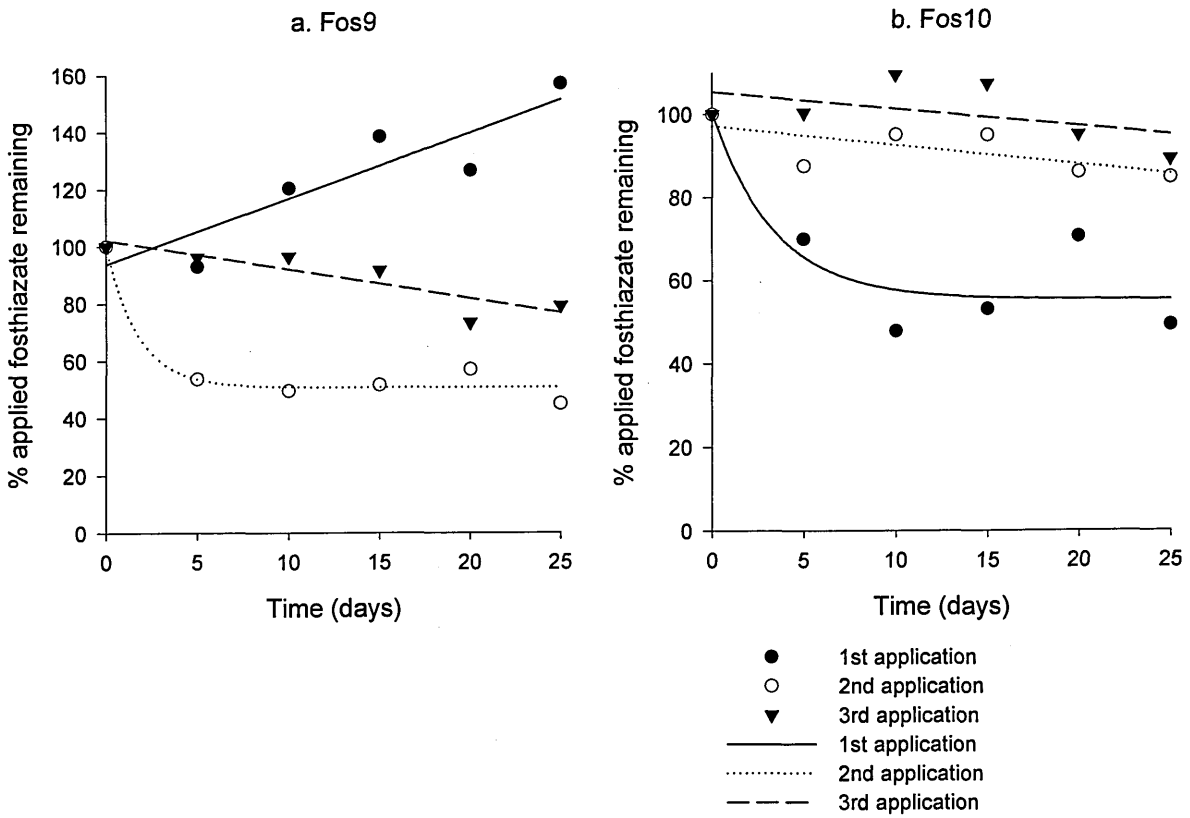


Figure 9 continued: Degradation of fosthiazate in soils FOS 9 and 10 after three successive applications. Data points are the mean (n=2) presented as a percentage of the day 0 concentration.

2.4 DISCUSSION

2.4.1 Oxamyl

One of the main purposes of this investigation was to attempt to induce enhanced degradation of oxamyl in soils that had been treated with oxamyl in the field but had not shown an efficacy problem. Oxamyl-degrading bacteria could then be isolated and identified from enhanced degrading soils (Chapter 3). Previous studies have established that enhanced rates of pesticide degradation are observed in previously treated soils (Suett, 1986; Smelt *et al.*, 1987; Karpouzas *et al.*, 1999a; Karpouzas *et al.*, 2001). This is thought to occur as a result of bacterial adaptation after the initial pesticide application, leading to

enhanced rates of degradation of subsequent applications because bacteria adapted to mineralise the pesticide are already present within the soil (Kearney and Kellogg, 1985; Felsot and Shelton, 1993). As such, it was suspected that repeated application in the laboratory could result in enhanced rates of degradation in soil samples that had not previously been reported as displaying a persistence problem in the field. It appears that in a number of the oxamyl soils this was successful. Oxamyl degradation was significantly faster ($p<0.1$ and 0.05) in the second and third applications than in the first in five of the ten soils investigated, suggesting that enhanced degradation has occurred in these soils after repeat application. This is consistent with other studies that have shown pesticide degradation to increase with subsequent applications of the chemical both in the laboratory and in the field (Smelt *et al.*, 1987; Cox *et al.*, 1996; Smelt *et al.*, 1996; Singh *et al.*, 2003b), although this is not always the case. The long-term study conducted by Bromilow *et al.* (1996) demonstrated enhanced degradation of aldicarb (also an oximecarbamate) after 20 years of application; however the rate of degradation of the fungicides benomyl and triadimefon andof the organophosphorus insecticide chlorfenvinphos were unaffected by the long treatment history with these chemicals.

Of the remaining five soils, four displayed rapid oxamyl degradation. Although differences between the applications were not significant, the DT50 values do show oxamyl to be degraded faster in the second and third applications in soils OX 4 and OX 7. Soil OX 10 demonstrated rapid degradation in all three applications from the outset, suggesting that adapted bacteria were already present (Torstensson, 1980; Felsot and Shelton, 1993). This soil had received in-field oxamyl applications in 2000 and 2001, prior to sampling in 2002, which could have resulted in the adaptation of bacteria to utilise oxamyl prior to the incubation study. Degradation of the second and third applications applied to OX 3 were slightly slower than in the first application as shown by the DT50 values; however degradation was still rapid in all three applications as the longest DT50 value was only

4.69 days. In addition, the standard error of the mean DT50 values for the second and third applications were quite large and the r^2 value for the third application fitted curve was low, indicating possible inaccuracies in the calculated DT50 values for this soil. In comparison, the degradation of oxamyl in soil OX 11 was slow in all three applications. The slow degradation observed in this soil can not be explained by any abiotic factor and as such may be due to a lack of adapted microorganisms present within the soil. Inoculating oxamyl-degrading bacteria, isolated from a different soil, into OX 11 could test this theory. Rapid oxamyl degradation would demonstrate that conditions within soil OX 11 were suitable for the growth of oxamyl-degrading bacteria, suggesting that adapted bacteria were not present within the soil (Singh *et al.*, 2003a).

The time taken to degrade 50% of an initial oxamyl concentration has previously been reported as 3.1 to 6.5 days in five Israeli soils with a pH greater than 7.0 (Gerstl *et al.*, 1984); 6.2 to 17.8 days in four soils at 15 °C, all of a pH greater than 7.0 (Bromilow *et al.*, 1980); 14 days in a loamy sand at 15 °C (Smelt *et al.*, 1979); and 11 and 15 days in a loamy sand and sandy soil respectively (Harvey and Han, 1978). The DT50 values reported in this chapter for the first oxamyl application applied to ten different soils ranged from 1.06 to 23.9 days and are therefore similar to previously published values. The largest DT50 value in this range, 23.9 days, occurred with the lowest pH soil, OX 5 (pH 5.9). However the rate of degradation in this soil greatly increased with subsequent applications so that by the third application the DT50 value had dropped to 2.6 days. In contrast, Singh *et al.* (2003b) failed to report increased rates of fenamiphos degradation with subsequent applications in low pH soils of 4.7, 5.7 and 6.7. A connection between pH and degradation rate was absent from soil OX 11 (pH 7.4), which demonstrated the slowest overall degradation of oxamyl and for which degradation data were erratic. This is inconsistent with previous research in which enhanced degradation of oxamyl has occurred in higher pH soils rather than in those of low pH (Smelt *et al.*, 1996). Of further interest is the peat

soil OX 8, which had a high organic matter content of 70%. The time taken to degrade 50% of the second and third applications is significantly (P<0.1 and 0.05) faster than that for the first application. This is surprising, as adsorption of oxamyl would be expected to be greater in this soil, thereby reducing the bioavailability of the chemical to soil microorganisms, slowing the rate of degradation (Walker, 1989; Weber *et al.*, 1993, Guo *et al.*, 1999). It has however, been shown that oxamyl is only weakly sorbed by soil (Bromilow *et al.*, 1980; Gerst, 1984). Soil OX 8 also has an intensive oxamyl application history dating back to the late 1970s. This intensive application history may have had a greater effect on the rate of degradation than the high organic matter content of the soil. Similarly, it is surprising that rapid oxamyl degradation was observed in soil OX 4 as this soil had been fumigated with 1,3-dichloropropene in the year prior to oxamyl application in the field. Soil fumigation has been shown to be effective at reducing enhanced degradation (Suett, 1986), although this is not always the case. The fumigant methyl bromide failed to reduce enhanced rates of cadusafos degradation nine months after fumigation. Rapid cadusafos degradation was however inhibited when the soil was fumigated only 20 days prior to cadusafos application (Karpouzas *et al.*, 2005a), suggesting that perhaps fumigation of soil OX 4 occurred too far in advance of oxamyl application to have an effect.

2.4.2 Aldicarb

As with oxamyl, one of the main aims of the soil incubation study was to investigate the potential for enhanced degradation of aldicarb in agricultural soils by applying three repeat applications in the laboratory. This was successful in two of the 15 soils, Ald 4 and Ald 15, as aldicarb total toxic residues were degraded significantly faster (P<0.1 and 0.05) in the second and third applications than in the first, suggesting that adapted microorganisms are involved in the degradation. Although not significantly different (probably because of the

low number of replicates and the large amount of variability between replicates), shorter DT50 values for the second and third applications were recorded in an additional four soils, Ald 6, 7, 10 and 14, suggesting that adapted soil microorganisms were also involved in the degradation of aldicarb and its oxidation products in these soils. An increase in the rate of degradation of aldicarb TTR with subsequent application is consistent with published research, where repeat aldicarb application in the laboratory or field has resulted in increased rates of degradation (Read, 1987; Smelt *et al.*, 1987; Bromilow *et al.*, 1996).

All the previously treated aldicarb soils received an aldicarb application in the field in the year prior to the incubation study. Soil Ald 6 had also been fumigated with 1,3-dichloropropene prior to aldicarb application in the field. Unlike soil OX 4, also fumigated in the field, the degradation rate of aldicarb was comparatively slow in the first application suggesting that 1,3 dichloropropene may have had an effect on the rate of degradation. However, subsequent doses were degraded much faster, demonstrating that 1,3-dichloropropene had not been totally effective at inhibiting the development of enhanced degradation within this soil. Ald 4, one of the fastest degrading soils within which second and third applications were degraded significantly faster ($P<0.05$) than the first, had previously been treated with oxamyl in the field in addition to aldicarb. There is evidence to suggest that pesticides of the same chemical group can enhance the degradation of other chemicals within that group, a phenomenon known as cross-enhancement (Smelt *et al.*, 1987; Suett and Jukes, 1988; Morel-Chevillet *et al.*, 1996). The previous oxamyl additions to this soil may have aided in the adaptation of soil microorganisms capable of rapidly degrading aldicarb.

Six of the aldicarb-treated soils (Ald 1, 2, 3, 5, 8 and 11) demonstrated slow rates of degradation of aldicarb TTR. A DT50 value of 29 days was recorded for the third application applied to Ald 1 compared to 1 day for Ald 4. Due to the incomplete

degradation of residues, particularly aldicarb sulphoxide, aldicarb TTR accumulated in these soils with successive applications. Four of these soils had pH values of less than 6.0. Aldicarb degradation has been shown to occur at a slower rate in soils with pH levels below 6.0 (Read, 1987; Smelt *et al.*, 1996). As such, the low pH of the majority of the slow-degrading soils may have contributed to the degradation rate.

A further contributing factor to three of these slow-degrading soils may have been high soil organic matter content. The three peat soils, Ald 2, 3 and 5, had organic matter contents of 70, 52 and 36% respectively. No increase in degradation with subsequent application was observed in these soils and the persistence of parent aldicarb and aldicarb sulphoxide residues were greater than that seen in the rapidly degrading soils. As previously mentioned in Section 2.4.1, high organic matter content often results in increased adsorption, leading to reduced bioavailability of the chemical and reduced degradation. The degradation of aldicarb and its oxidation products has been shown to proceed at a slower rate in a high organic matter content peaty sand soil, within which the half-life of aldicarb sulphoxide was measured as 46 days (Smelt *et al.*, 1978 a, b and c). Guo *et al.* (1999) monitored the degradation of aldicarb in soils amended with different amounts of activated carbon and found adsorption to increase with increasing amounts of activated carbon, which in turn resulted in decreased degradation rates. In contrast, Bromilow *et al.* (1980) reported rapid aldicarb oxidation in a sandy loam soil amended with peat as well as in a lower organic matter sandy loam. The organic matter content of the peat amended soil was however far lower than that found in Ald2, 3 and 5. Unlike previous studies, in which the slow rate of degradation of aldicarb sulphoxide has been found to be largely responsible for the overall slow degradation of aldicarb TTR (Smelt *et al.*, 1987, Suett and Jukes, 1988; Bromilow *et al.*, 1996; Smelt *et al.*, 1996), both parent aldicarb and aldicarb sulphoxide were seen to be degraded slowly in the three high organic matter soils. The slow oxidation of parent aldicarb led to continually rising aldicarb sulphoxide

concentrations over the 25 day period and this was unaffected by successive applications. The degradation pattern of aldicarb and its oxidation products in the remaining three slow-degrading soils, that contained a much lower percentage of organic matter, was in line with previous research as parent aldicarb was oxidised rapidly to aldicarb sulphoxide, which in turn was much more persistent. Thus provides further evidence that increased adsorption due to high organic matter content may have had an effect on the rate of aldicarb oxidation.

A clear difference between the slow-degrading soils, those that demonstrated enhanced degradation rates with repeat application and the three soils that displayed rapid degradation of all applications can be seen when the group's mean DT50 is calculated for each application. Mean DT50 values for the first application were 11.7 (n=2) (soil Ald 11 was not included), 8.2 (n=5) and 5.4 (n=2) days for the slow, enhanced and rapidly degrading soils respectively. It should be noted that DT50 values for the first application could only be calculated for two of the slower-degrading soils and as such this value is not truly representative of all the slow-degrading soils. By the second application the difference was far greater. The mean DT50 value for the slow-degrading soils was calculated as 20.9 days (n=3) whereas that for the enhanced and rapidly degrading soils was 3.4 (n=6) and 5.5 (n=2) days respectively. Similarly, a large difference was seen in the third aldicarb application. Mean DT50 values were calculated as 18.7 (n=3), 1.7 (n=5) and 1.6 (n =2) days for the slow, enhanced and rapidly degrading soils respectively. The first application DT50 values for the degradation of aldicarb toxic residues are consistent with other studies investigating the degradation of aldicarb in soil incubation. Smelt *et al.* (1988) reported DT50 times of 5-17 days in soils previously treated with aldicarb and 25-50 days in the slower-degrading previously untreated soils. In a later study Smelt *et al.* (1996) recorded half-lives of 2-3 days in soils previously treated with aldicarb three times in the field. Similarly, Read (1987) recorded complete degradation of aldicarb residues within 1-4 weeks in previously treated soils. In the long-term study by Bromilow *et al.*

(1996), complete degradation of aldicarb toxic residues took 30 days in soils that had previously received in-field aldicarb treatments for 20 years. With the exception of soil Ald 1, the percentage of applied aldicarb ultimately oxidised to aldicarb sulphone was rarely more than 15% in all soils tested and aldicarb sulphone appeared to have little effect on the TTR degradation rate. There also appeared to be no connection between repeat application and aldicarb sulphone concentration. The conversion of applied aldicarb to aldicarb sulphone varies greatly in published research, ranging from undetected in a previously treated soil (Smelt *et al.*, 1987) to 50-73% in a sandy loam (Bromilow *et al.*, 1980). Suett and Jukes (1988) reported less than 6% conversion of applied aldicarb to aldicarb sulphone in four previously treated soils and 10-28% conversion in a previously untreated soil. The aldicarb sulphone concentrations reported in this study do appear to fit in at the lower end of this range.

2.4.3 Fosthiazate

Fosthiazate degradation data were erratic and a large amount of variation was observed between duplicate samples, which made calculating DT50 values and statistically analysing differences between applications difficult. As such, few DT50 values were available and significant differences have not been calculated.

The main aim of the experiment was to investigate the potential for enhanced fosthiazate degradation within agricultural soils by applying three successive applications within the laboratory. However, enhanced degradation was not observed in any of the ten soils tested. Slow degradation was observed in all soils and fosthiazate residues accumulated as successive applications were applied to soils within which the previous application had not been fully degraded. The time taken to degrade 50% of the third fosthiazate application was greater than 60 days in some soils.

All samples had been treated with fosthiazate in the field a year prior to the incubation study. However, in addition to this, five of the ten soils had also been fumigated with 1,3-dichloropropene. As mentioned in the previous section, fumigation has been shown to inhibit enhanced degradation; however as slow degradation was also observed in the five soils that were not fumigated in the field, it is difficult to determine whether the 1,3-dichloropropene application had any effect on the rate of degradation. A further factor that could potentially have affected the rate of degradation is the high organic matter content of five of the soils. With percentage organic matter contents of 20% or more in these soils, it would be suspected that increased adsorption would occur, causing reduced availability of the chemical and thus a slower rate of degradation (Walker, 1989; Weber *et al.*, 1993, Guo *et al.*, 1999). However, as with the effect of soil fumigation, the five soils that had comparatively low organic matter contents also demonstrated slow fosthiazate degradation with the accumulation of residues upon repeat application.

Very little research has previously been conducted into the degradation of fosthiazate in soils; however, the study by Qin *et al.* (2004) reported half-lives (based on first-order degradation kinetics) of 17.7 and 26.8 days in two sandy loam soils and 46.8 days in a clay loam with a lower pH of 5.5. The authors reported a pH effect on the persistence of fosthiazate; persistence time was seen to decrease with increasing soil pH. This may also be true for the incubation study reported in this chapter, as comparatively short DT50 values of 19.8, 17, and 16.8 days for the first, second and third applications respectively, were recorded for the highest pH soil, Fos 3 (pH 7.6). It is however, difficult to determine whether this is true for all soils tested because of the lack of calculated DT50 values and the erratic nature of the data.

Other organophosphorus pesticides, such as the nematicides cadusafos (Karpouzas *et al.*, 2004), fenamiphos (Ou *et al.*, 1993) and ethoprophos (Karpouzas *et al.*, 1999a) and the

insecticide chlorpyrifos (Singh *et al.*, 2003a), have been shown to be susceptible to enhanced degradation. Therefore, the potential for enhanced degradation of fosthiazate within agricultural soils cannot be ruled out. At the time of the incubation study fosthiazate was a relatively new pesticide and as such had not been used extensively in the field. Bacterial adaptation to rapidly degrade the nematicide might still be in its infancy, but if other organophosphates are used as an example, enhanced degradation of fosthiazate could pose a problem in the future.

The degradation data in general for all three nematicides were erratic with large differences between replicates in a number of cases. The cause of this is almost certainly due to inadequately mixing the nematicide into the soil samples. Nematicide solutions were applied to the samples after the soil moisture content had been increased by the addition of sterile distilled water. As such, this addition of water could not be used to aid percolation of the chemical through the soil, as is done in other incubation studies (Bromilow *et al.*, 1980; Suett *et al.*, 1993; Walker *et al.*, 1993; Walker and Austin, 2004). Sieving the soil sample after addition of the chemical and moisture content adjustment is a further technique used by researchers (Karpouzas *et al.*, 2001; Singh *et al.*, 2002; Singh *et al.*, 2003a). However, this method would have been impractical due to the large number of samples to process, and could also have increased the chances of soil cross-contamination and experimenter exposure to the chemicals. To ensure adequate mixing of oxamyl into the soil samples used in the soil incubation study in Chapter 4, the soil moisture content was adjusted after the addition of the nematicide.

One of the main aims of the incubation study was to induce enhanced degradation of the nematicides aldicarb, oxamyl and fosthiazate in soils, so that bacteria involved in enhanced degradation could then be isolated. To increase the chances of finding soils that demonstrated enhanced degradation, a large number of soils were tested (35 in total). As a

result it was only possible to analyse duplicate samples for each soil. Duplicate replication of soil samples in incubation studies is not uncommon (Read, 1987; Suett and Jukes, 1988; Walker and Welch, 1992; Bromilow *et al.*, 1996; Cox *et al.*, 1996); however, by increasing the number of replicates a greater degree of accuracy of the DT50 values may have been achieved and a higher percentage of variance accounted for by the fitted curves. All soils were analysed in triplicate in the soil incubation study detailed in Chapter 4. Similarly, the use of sterilised soils as 'controls' to establish the relative contributions of abiotic and microbial factors on the rate of degradation of the three nematicides may have been of value, particularly for those soils in which the rate of degradation did not change with subsequent applications (Qin *et al.*, 2004). However, this too would have meant that fewer soils could have been examined, possibly reducing the chances of finding soils with the potential for enhanced degradation.

The occurrence of enhanced degradation, as observed as an increase in the rate of degradation with successive application in the laboratory, cannot be transferred directly to the field situation because the short intervals between applications in this study would not occur in the field. Also, abiotic factors such as soil temperature and moisture content that were controlled in the laboratory would have an effect in the field. However, the results from this study have demonstrated that there is potential for enhanced degradation of oxamyl and aldicarb in agricultural soils as a result of intensive treatment, providing evidence for the ability of soil bacteria to adapt in some way to rapidly degrade these two carbamate nematicides.

2.4.4 Conclusions

- The potential for enhanced degradation of the nematicides oxamyl and aldicarb was observed in a number of geographically different soils.

- Increased rates of degradation of oxamyl and aldicarb were observed with repeat application, suggesting that adapted bacteria are involved in the degradation.

- The degradation of fosthiazate was slow in all soils tested regardless of soil type and treatment history. Fosthiazate residues were seen to accumulate within the soils with each subsequent application.

3. ISOLATION AND IDENTIFICATION OF OXAMYL-DEGRADING BACTERIA

3.1 INTRODUCTION

It is now well established that soil microogranisms are responsible for the enhanced degradation of pesticides, a phenomenon that is becoming more and more prevalent in soils after repeated application of the same or similar compounds (Felsot and Shelton, 1993).

A number of methylcarbamate-degrading bacteria have been isolated and these cover a range of bacterial genera, including *Achromobacter* (Karns *et al.*, 1986), *Pseudomonas* (Bano and Mussarrat, 2004), *Flavobacterium, Chrysobacterium* (Karpouzas *et al.*, 2000a), *Aminobacter* (Desaint *et al.*, 2000) and *Sphingomonas* (Kim *et al.*, 2004). Some studies have isolated similar pesticide-degrading bacteria from different geographical locations (Parekh *et al.*, 1994; Rousseaux *et al.*, 2001). Karpouzas *et al.* (2000a) isolated carbofuran-degrading bacteria from soils in the UK and Greece. Using a number of molecular techniques, they were able to identify the isolates and determine the degree of similarity between them. Their results indicated that closely related carbofuran-degrading bacteria could be isolated from geographically distinct soils.

Predicting the persistence time of a pesticide after application to a field soil would have economic benefits for growers. Specific pesticide-degrading species within the soil could potentially be used as an indicator for enhanced degradation. Although this may be possible in some cases, such as those mentioned above, it has been shown that bacterial species responsible for the degradation of the methylcarbamate carbofuran can vary both between and within soils. A high degree of phenotypic and genotypic diversity has been reported amongst carbofuran-degrading bacteria isolated from French and English soils (Parekh *et al.*, 1995; Desaint *et al.*, 2000).

A further possibility for predicting pesticide persistence lies with the quantification of specific degradation genes within the soil, such as the methylcarbamate degradation gene (*mcd* gene). The *mcd* gene was initially identified in *Achromobacter* sp. WM111, a carbofuran-degrader (Tomasek and Karns, 1989). Strain WM111 was able to degrade carbaryl and aldicarb in addition to carbofuran. Similarly, *mcd*-gene homologous sequences were found in a carbofuran-degrader, designated strain ER2, isolated from a Canadian soil (Topp *et al.*, 1993). This phylogenetically unique organism was able to rapidly degrade not only carbofuran but also the carbamates carbaryl and bendiocarb. The *mcd* gene is therefore not totally specific to carbofuran and thus may be present in bacteria capable of degrading the oximecarbamate nematicide oxamyl. However, the *mcd* gene is not always detected in methylcarbamate-degrading bacteria (Desaint *et al.*, 2000), indicating the involvement of other, as yet unidentified, genes. Screening oxamyl-degrading strains isolated from geographically distinct soils for *mcd*-gene homologous sequences may give an idea of the occurrence of the *mcd* gene amongst oxamyl-degraders.

Genes involved in the degradation of pesticides are often found to be encoded on plasmids. These self-replicating, circular pieces of DNA are independent of chromosomal DNA and thus pose an evolutionary advantage (Kearney and Kellogg, 1985; Karns, 1990). Topp *et al.* (1993) found their carbofuran-degrading bacteria, strain ER2, to contain a similar-sized plasmid (pER2a, 120 kb) to the *mcd*-gene harbouring plasmid (plasmid pPDL11, >100 kb) from *Achromobacter* sp. WM111. The 120-kb plasmid from ER2 also contained sequences homologous to the *mcd* gene. Using the whole sequence of plasmid pPDL11 as a plasmid probe, Parekh *et al.* (1996) found homology with similar-sized plasmids from 24 *mcd*-gene homologous isolates. Loss of degrading ability in the absence of the target chemical can indicate that the genes involved are located on plasmids (Head *et al.*, 1992). Strains that have lost their degrading ability are often found to be missing plasmids that are present in strains still able to degrade the chemical (Head *et al.*, 1992; Hayatsu *et al.*, 2000).

3.1.1 Objectives

- To isolate oxamyl-degrading bacteria from soils that displayed enhanced oxamyl degradation in a previous soil incubation study (Chapter 2).

- To characterise the oxamyl-degrading strains.

- To screen oxamyl-degrading isolates for the presence of the *mcd* gene.

- To investigate whether oxamyl-degrading ability is lost after repeated sub-culture in the absence of oxamyl, indicating the involvement of plasmids.

3.2 MATERIALS AND METHODS

3.2.1 Preliminary Experiment: Media Test

The objective of this experiment was to determine whether the microorganisms involved in the degradation of oxamyl required a carbon or nitrogen source, or soil for degradation of oxamyl. The degradation rate of oxamyl by soil OX 10 was examined in mineral salts media (MSM) supplemented with oxamyl (MSM+OX); MSM supplemented with nitrogen and oxamyl (MSMN+OX); MSM supplemented with glucose and oxamyl (MSMG+OX); and soil extract media (SEM).

3.2.1.1 Media Preparation

Media supplemented with a carbon or nitrogen source contained either NH_4Cl or glucose (1.0 g l^{-1}) as well as the following salts (in g l^{-1}) to make up the Mineral Salts Medium (MSM): KH_2PO_4, 2.27; $Na_2HPO_4.12H_2O$, 5.97; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $MnSO_4.4H_2O$, 0.02; $FeSO_4$, 0.005; NaCl, 1.0 (Roberts *et al* 1991). Media supplemented with nitrogen (MSMN) did not contain NaCl. Stock solutions were made following the method of Roberts *et al.* (1991). Ten times the stated amount of KH_2PO_4, $Na_2HPO_4.12H_2O$ and NH_4Cl/ NaCl (NaCl was used in MSM and MSM + glucose only) were dissolved together in 1 litre of distilled water and autoclaved at 121°C for 15 min. After cooling, the correct amount of stock was added to sterile distilled water (SDW) to give the required concentration of each salt. Similarly, ten times the stated amount of $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and $MnSO_4.4H_2O$ were dissolved together in 1 litre of distilled water and autoclaved. After cooling, the correct amount of stock was added to the media to give the concentration stated above. The correct amount of $FeSO_4$ was added to the media as part of a 50 times stock solution in distilled water that had been filter sterilised (0.2 μm pore size). For the MSMG media, MSM was made as above and glucose was added to the media as a filter sterilised (0.2 μm pore size), ten times stock solution in distilled water. The pH of the

mineral salts media was approximately 6.8. Soil Extract Media (SEM) was made from air-dried, sieved (2 mm mesh) soil (soil OX 10 was used) and distilled water at a weight to volume ratio of 1:1. The soil solution was mixed by hand shaking prior to autoclaving at 121°C for 30 min to sterilise. The solution was then centrifuged to remove large particles and the supernatant autoclaved at 121°C for a further 30 min.

To make solid MSMN+OX, the above sterile stock solutions were added to sterile, molten bacteriological agar (15 g l^{-1}), held at approximately 50°C, to give the correct g l^{-1} concentrations. Oxamyl was added to the sterile, molten media as a solution in 50:50 methanol and SDW (150 mg l^{-1}) to give a final concentration of 15 mg l^{-1}.

3.2.1.2 Media Test

Each media was dispensed (20 ml) into sterile, 100-ml Duran style bottles. Oxamyl was added to all enrichment culture broths as a solution in 50:50 methanol and sterile distilled water (150 mg l^{-1}) to give a final concentration of 15 mg l^{-1}. This is approximately five times greater than the commercial application of oxamyl in the field. Each enrichment media type was inoculated with 0.5 g of soil OX10 and replicated three times. Three replicates of uninoculated control broths of media MSM+OX, MSM+OX+Glucose and SEM+OX were included to monitor the abiotic rate of degradation. All cultures were incubated at 25°C in a shaking incubator at 120 rpm. Cultures were sampled immediately after inoculation to determine the initial oxamyl concentration and at approximatly 2-day intervals from then on. A 0.5 ml sub-sample was removed from the culture and mixed with an equal amount of methanol to stop microbial degradation. Sub-samples were centrifuged briefly to remove solid matter before analysis by HPLC (Section 3.2.2.1) to determine the remaining oxamyl concentration.

3.2.2 Initial Enrichment Culture Method

Soils demonstrating enhanced degradation of oxamyl in the incubation study (soils OX 4, OX 7 and OX 10) were taken on to enrichment culture stage to enable isolation of the bacteria involved. The enrichment culture method followed that described previously by Roberts *et al.* (1991) and Cox *et al.* (1996). Sub-samples (500 mg) of soil were taken, each of which had received three successive doses of oxamyl in the incubation study and had since been stored at –20°C. These sub-samples were used to inoculate 20 ml of Mineral Salts Media supplemented with nitrogen (MSMN) in sterile, 100-ml bottles. Oxamyl had been added to the media as a solution in 50:50 methanol and sterile distilled water (150 mg l^{-1}) to give a final concentration of 15 mg l^{-1} (MSMN+OX). All enrichment cultures were incubated at 25°C in a shaking incubator at 120 rpm with the lids screwed down. Cultures were sampled (0.5 ml) immediately after inoculation and then periodically. An equal amount of methanol was added to the sub-sample to stop microbial degradation. Sub-samples were centrifuged briefly to remove solid matter before analysis by HPLC (Section 3.2.2.1).

When the oxamyl concentration in the cultures had decreased to below 50% of the initial concentration, 0.5 ml of culture was transferred into fresh bottles containing 20 ml MSMN+OX (15 mg l^{-1}). At the point of 50% degradation in the second enrichment culture cycle, 0.5 ml of culture was again transferred into bottles containing 20 ml of fresh MSMN+OX, making three enrichment cycles in total (Figure 10). There were three inoculated replicates per soil and three uninoculated control broths for each of the three incubation cycles. Sub-samples for analysis of oxamyl concentration were taken immediately after inoculation of fresh MSMN+OX and at intervals thereafter (sampling procedure as above).

3.2.2.1 HPLC Analysis

Oxamyl analysis was carried out on a Hewlett Packard Series 1100 HPLC using a Phenomenox, 250 x 46 mm Sphereclone 5 μm ODS column held at 30°C. The mobile phase was 50:50 HPLC grade water and methanol run at a flow rate of 1.2 ml min^{-1}. A 20-μl injection was monitored at 220 nm with a retention time of 3.5 min.

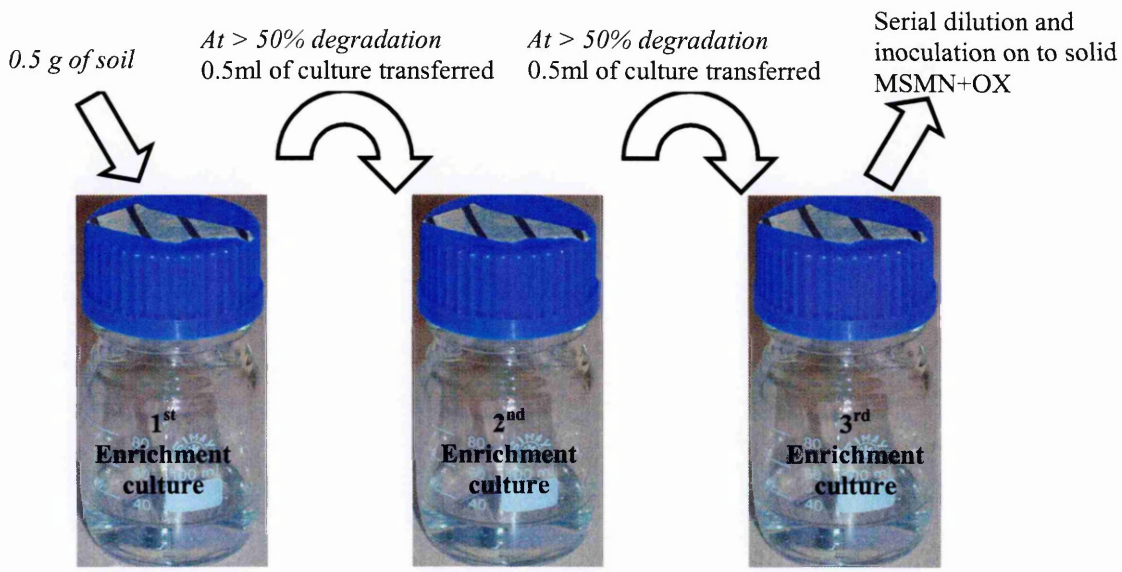


Figure 10: Diagram of the enrichment culture method

3.2.3 Initial Attempt to Isolate Oxamyl-Degrading Bacteria

After the oxamyl concentration had decreased to below 50% in the third enrichment cycle, 10-fold serial dilutions were prepared in 9 ml of MSMN (no oxamyl), and 0.1 ml of each dilution was spread onto solid MSMN+OX agar plates. Plates were incubated at 25°C for approximately 6 days.

Approximately ten well-isolated colonies per enrichment culture replicate were picked off the highest dilution plates showing growth and sub-cultured by streaking onto MSMN+OX agar plates. The number of colonies taken from dilution plates was often dependent upon

the number of different colony types present. If only a few different colony types were observed, then fewer colonies were sub-cultured; however, in most cases at least 30 colonies were sub-cultured per soil. All plates were incubated at 25°C for approximately 6 days, or until visible colonies had formed. In the majority of cases colonies remained very small on MSMN+OX agar plates.

3.2.4 Test of Degrading Ability

After incubation, isolates that had been sub-cultured from dilution plates were tested for their ability to degrade oxamyl. From each plate approximately five identical, well-isolated colonies were picked off and inoculated into 1 ml SDW to create a suspension, which was used to inoculate 20 ml MSMN+OX (15 mg l^{-1}) in 100-ml bottles. All test cultures were incubated at 25 °C with shaking at 120 rpm along with three uninoculated MSMN+OX control broths. Test cultures were sampled periodically to determine the oxamyl concentration (Sections 3.2.2 and 3.2.2.2).

3.2.5 Preliminary Investigation: Does Methanol Provide an Additional Carbon Source in the Media?

The media was designed so that the added oxamyl provides the sole carbon source; therefore excessive growth of bacteria unable to utilise oxamyl as a carbon source should not occur. However, isolation of degraders from enrichment cultures proved difficult because of the high number of non-degrading bacteria present on MSMN+OX agar plates. Another carbon source must, therefore, have been present in the media. An experiment was conducted to determine whether the small amount of methanol, added to the media as part of the 50:50 methanol and water oxamyl stock solution, could provide an additional carbon source.

Enrichment culture broths were set up as for an enrichment culture study (Section 3.2.2), except for differences in methanol and oxamyl content:

1. 20 ml MSMN containing 15 mg l^{-1} oxamyl as a solution in 50:50 methanol and water
2. 20 ml MSMN containing the same amount of a 50:50 methanol and water solution as media 1 but **without** oxamyl
3. 20 ml MSMN only, no methanol or oxamyl
4. Uninoculated MSMN control broths containing 15 mg l^{-1} oxamyl as a solution in 50:50 methanol and water.

All broths except controls were inoculated with a 1 ml suspension (sterile distilled water) of a bacterial colony type isolated from soil OX 10 that had demonstrated rapid oxamyl degradation in test culture. All culture types were replicated three times and incubated at 25°C in a shaking incubator at 120 rpm in the dark. Cultures were visually assessed for turbidity.

3.2.6 Enrichment Culture Final Method

3.2.6.1 Enrichment Culture Modified Method: Elimination of Methanol

To overcome the effect of methanol and reduce the number of non-degrading bacteria present in enrichment cultures, the media preparation procedure used to enrich, isolate and maintain oxamyl-degrading bacteria was modified. Firstly, a much more concentrated stock solution of oxamyl was made at 5 g l^{-1}. The solution was still made up in 50:50 methanol and water because the methanol aids the solubility of oxamyl. However, because of the higher concentration a much smaller amount of the solution gave the required final concentration of 15 mg l^{-1}. In order to completely eliminate the now much smaller amount of methanol, 60 μl of the 5 g l^{-1} oxamyl solution was added to empty, sterile, 100-ml bottles. These were then left in a microbiological safety cabinet, uncapped, for at least 30

minutes to allow the methanol to evaporate. MSMN (20 ml) was then added to the bottles and mixed by hand shaking, resulting in a final oxamyl concentration of 15 mg l^{-1}. Oxamyl now provided the only possible carbon source in the medium as demonstrated by much less turbid growth.

To remove methanol from solid media, 1.5 ml of the 5 g l^{-1} oxamyl solution was added to 500 ml of molten MSMN (bacteriological agar 15 g l^{-1}) that had been cooled to approximately 50°C. The bottle of agar was then left, uncapped, in a water bath (50°C) inside the microbiological safety cabinet for at least 1 hour to allow the methanol to evaporate. Apart from the change to the media preparation, the three enrichment culture cycles remained as described in Section 3.2.2. All relevant media were now prepared using the methanol elimination procedure.

3.2.6.2 Enrichment Cultures without Methanol

Enrichment culture studies were repeated using the modified method, eliminating methanol from the medium. Enrichment studies were initially carried out with soils OX 4, OX 7 and OX 10 and apart from the changes affecting the methanol content the method remained the same (Section 3.2.2), with three enrichment cycles followed by serial dilution and inoculation onto MSMN+OX agar plates. Once enrichment culture studies with these soils had been completed, further soils that had demonstrated enhanced oxamyl degradation in the incubation study were taken on to enrichment culture. All soil sub-samples came from soils that had received three successive applications of oxamyl in the incubation study and all soils had been stored at –20°C since completion of the incubation study. Soils OX 2, OX 3, OX 5, OX 9 and OX 12 were tested. Soil OX 11 was also tested in enrichment culture, although this soil had not demonstrated enhanced degradation of oxamyl in the incubation study.

3.2.7 Isolation and Cryogenic Storage of Oxamyl-Degrading Bacteria

All procedures followed those already described in Section 3.2.2. Ten-fold serial dilutions of the third enrichment cycle cultures were made by inoculating 1 ml of culture into 9 ml MSMN (no oxamyl) and spreading 0.1 ml of each dilution on to MSMN+OX agar plates (made following the methanol elimination method). After incubation at 25°C for approximately 6 days, ten well-isolated colonies were taken, per replicate, from the highest dilution plates showing growth and streaked on to MSMN+OX agar plates. The number of colonies taken from dilution plates sometimes depended upon the number of different colony types present. If only a few different colony types were observed, then fewer colonies were sub-cultured, although in most cases at least 30 colonies were sub-cultured per soil. Test cultures were set up as before (Section 3.2.4) to test the degrading ability of isolates; however, the modified method was followed eliminating methanol from the medium.

If test cultures demonstrated rapid oxamyl degradation, a loop full of culture was streaked on to both MSMN+OX agar and Nutrient Agar (Oxoid) plates and incubated at 25°C. The remainder of the culture was kept and stored at 4°C. Nutrient Agar plates were used to check the purity of the culture and were incubated for approximately 4 days. MSMN+OX plates were incubated for approximately 6 days and were used to supply colonies for the cryogenic culture collection. To ensure as many oxamyl degraders as possible could be identified, colonies growing on MSMN+OX plates were picked off regardless of purity on NA plates and cryogenically stored. A single well-isolated colony was taken from each MSMN+OX plate; however if more than one colony type was visible, all colony types were picked off and stored separately. If, as in some cases, different colony types could not be separated, both types were stored together. Isolates were inoculated into Protect Vials containing ceramic beads in a cryopreservation liquid (Technical Service Consultants Ltd) and stored at –80°C following the manufacturer's instructions.

3.2.7.1 Purifying Oxamyl-Degraders

Non-pure but positively degrading cultures were taken on to determine which colony type was responsible for oxamyl degradation. A single bead was taken from each Protect Vial and inoculated directly into 20ml MSMN+OX (15 mg l^{-1}) and incubated at 25°C with shaking at 120 rpm. Sub-samples were taken periodically and analysed by HPLC to determine whether rapid oxamyl degradation was occurring. For rapidly degrading cultures only, a loop-full of culture was streaked on to MSMN+OX agar and NA plates and incubated at 25°C for approximately 4-6 days depending on the media type (Section 3.2.3.1). NA plates were checked to ensure that the culture was pure. If the culture rapidly degraded oxamyl but was still not pure as determined from the NA plates, one more attempt would be made to distinguish which colony type was responsible. The different colony types were picked off the MSMN+OX agar plates and re-tested by inoculating directly into separate 20 ml MSMN+OX (15 mg l^{-1}). The test culture procedure was repeated as above and a loop-full of rapidly degrading culture was streaked on to MSMN+OX agar and NA plates and incubated as before. If the oxamyl-degrading culture was now pure, a colony would be taken from the MSMN+OX plate, inoculated into a Protect Vial and stored at –80 °C (Section 3.2.3.1).

3.2.8 Characterisation of Oxamyl-Degrading Isolates

3.2.8.1 DNA Extraction and 16SrRNA Gene Amplification

Media Preparation

For the DNA-extraction procedure, isolates were cultured in MSMN+G+OX. This media consisted of MSMN+OX (15 mg l^{-1}) containing glucose as an additional carbon source at 1 g l^{-1} (Section 3.2.1.1). The additional carbon source was added to enable greater bacterial growth thereby giving higher yields of DNA during extraction.

DNA Extraction

For all characterisation procedures, cryogenically stored isolates were used. A single bead was taken from Protect Vials and inoculated directly into 20 ml MSMN+G+OX and incubated at 25°C with shaking at 120 rpm until turbid growth was observed, approximately 2-4 days. During incubation, 0.5 ml sub-samples were taken from the culture and analysed by HPLC to ensure that the isolate still rapidly degraded oxamyl. After incubation the culture was left to stand before transferring approximately 15 ml into a sterile 20-ml Universal tube, being careful not to transfer any salt crystals that may have formed and settled at the bottom of the culture. The 15 ml was then centrifuged (4000 *g* for 15 min) to pellet the cells. The supernatant was disposed of and the pellet stored at –20°C. The remaining 5 ml of MSMN+G+OX culture was used to inoculate NA plates so that culture purity could be confirmed. Bacterial DNA was extracted from the pelleted cells after defrosting at room temperature for approximately 20 min. To extract the DNA, the Promega Wizard Genomic DNA Purification Kit was used according to manufacturer's instructions. Extracted DNA was stored at 4°C and used for both 16S rRNA and *mcd* gene amplification.

PCR Amplification of the 16SrRNA Gene

Two PCR primers were used to amplify approximately 1,300 base pairs (bp) of a universal 16S rRNA gene: forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al* 1998). PCR reactions were carried out in a total reaction volume of 25 μl, containing: 100 μM concentration of each nucleotide, 100 nM of each primer, 20 U of Red Hot *Taq* polymerase (Abgene, Epsom, United Kingdom) per ml, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μg of gelatin per ml, 0.5 mg of Tween 20 (Sigma) per ml, 0.5 mg of Nonidet P-40 (Sigma) per ml, 15.3 μl of PCR water and 5 μl of DNA sample. Negative controls contained 5 μl of water instead of sample DNA. DNA amplification was performed on a

PTC-100 thermal cycler (MJ Research). The following programme was used: 30 cycles of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 45 sec with an initial melt of 94°C for 2 min and a final extension step of 5 min at 72°C. PCR products were analysed by agarose gel electrophoresis on a 2% gel and stained with ethidium bromide (0.5 μg ml) in order to visualise DNA bands.

In order to extract and purify the PCR products, samples displaying strong bands were visualised in low-melting point agarose gel (1%) stained with ethidium bromide (0.5 μg ml), with five replicates per sample to ensure enough DNA was obtained. The PCR products were excised from the gel by cutting with a sharp scalpel. Gel slices were dissolved and the DNA purified using a QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. Purified DNA was eluted in water and quantified after gel electrophoresis using a Gel Doc 1000 gel documentation system and a serial dilution of Lambda DNA (Promega) standards from 0.1 to 100 ng μl. A volume of each PCR product containing 120 ng of DNA was air dried and sent for sequencing to MWG Biotech (Germany). Sequences were compared to the bacterial sequences on the Genbank DNA sequence library using BLASTN software (Altschul *et al.*, 1997) on Biology Workbench version 3.2 (http://workbench.sdsc.edu).

3.2.8.2 Microbiological Characterisation Methods

Maintenance of Isolates

For all tests, cryogenically stored cultures were used. A single bead was taken from Protect Vials and streaked directly on to NA plates. Plates were incubated at 25°C until colonies had formed a good size, which took approximately 2-4 days. Colonies were taken from NA plates for use in the various characterization tests.

Gram Reaction Test

Well-isolated colonies were picked off NA plates and fixed on to glass slides by smearing in a drop of SDW and air-drying. Fixed slides were then covered in crystal violet and left for 60 seconds before rinsing with tap water. A 10% iodine solution was then applied to the slide and again left to stand for 60 seconds. This was rinsed away with tap water before covering the slide with a 95% alcohol solution. This was repeatedly rinsed and reapplied to the slide until the water ran clear. In the final step, safranin was applied to the slide and left for 60 seconds before rinsing and finally blotting dry (Gerhardt, 1994). Positive (*Staphylococcus epidermidis*) and negative (*E. coli*) quality controls were included with the samples. Slides were viewed using the oil-immersion lens of a light microscope.

Catalase Test

Well-isolated colonies were picked off NA plates and smeared in a droplet of 3% hydrogen peroxide on a glass slide. Production of gas bubbles was taken as a sign of catalase activity (Gerhardt, 1994). *Staphylococcus epidermidis* was used as a positive control.

Oxidase Test

Well-isolated colonies were picked off NA plates and smeared onto filter paper to which a few drops of 1% tetramethyl-*p*-phenylenediamine had been applied. The production of a purple colour indicated oxidase activity (Gerhardt, 1994). *Pseudomonas fluorescens* was used as a positive control and *E. coli* as a negative control.

API 20NE

Initially, isolates were tested for enzyme production and utilisation of sugars using API 20NE test strips (BioMerieux). Isolates were taken directly from NA plates and inoculated into the strips following manufacturer's instructions. The incubation procedure was

however changed to 25°C for 48 h (Roberts *et al.*, 1998). *Pseudomonas fluorescens* was used as a positive control. Results were checked using BioMerieux software.

3.2.9 PCR Detection of the Mcd *Gene in Oxamyl-Degrading Isolates*

To increase the chances of detecting sequences homologous to the *mcd* gene, two different sets of primers were used: those described by Parekh *et al.* (1996) (primer set one) and those by Derk *et al.* (2003) (primer set two).

3.2.9.1 Mcd-*Gene Detection Using Primer Set One (Parekh et al., 1996)*

The DNA-extraction method was as described in Section 3.2.8.1. Extracted DNA was amplified using *mcd*-gene specific primers and the PCR detection method closely followed that by Parekh *et al.* (1996). Forward and reverse primers were used to amplify a 561 bp fragment of the *mcd* gene, forward primer (*mcd*L1) (5'-CAA GAA CTC AAA TCC ATC TAC CTT GCC C-3'); reverse primer (*mcd*R1) (5'-ATC CTT CCC TCG GAA TGA ATC GTC TCG-3'). PCR was performed using a DNA Thermal Cycler (PTC-100, MJ Research). PCR reaction mixtures containing 5 μl of DNA sample were as described previously in Section 3.2.8.1. Negative controls contained 5 μl of water instead of sample DNA. Positive controls contained 5 μl of DNA from plasmid pPDL11 from the carbofuran degrader *Achromobacter* sp. strain WM111 (kindly donated by Nisha Parekh). The following programme was used: 30 cycles of 94°C for 1 min, 61°C for 1 min, and 74°C for 1 min with an initial melt of 94°C for 2 min and a final extension step of 5 min at 74°C. PCR products were analysed by agarose gel electrophoresis on a 2% gel stained with ethidium bromide (0.5 μg ml) in order to visualise DNA bands.

3.2.9.2 Mcd-*Gene Detection Using Primer Two (Derk et al., 2003)*

The majority of isolates were also tested using a second set of primers to improve the chances of detecting the *mcd* gene. This method closely followed that by Derk *et al.* (2003). Again, the DNA-extraction method was as described in Section 3.2.8.1. Primer set two amplified a 406 bp fragment of the *mcd* gene. The forward primer was designated P391 (5'-GGT ACC GGG CGT GGC TCG ATC ATG-3') and the reverse primer P9 (5'-CCC CTC GGA ATG AAT CGT CTC GGC-3'). The procedure was carried out using the same reaction mix and thermal cycler as above with a programme of: 30 cycles of 94°C for 1 min, 65°C for 1 min, and 74°C for 1 min with an initial melt of 94°C for 3 min and a final extension step of 5 min at 74°C. Negative (sterile Milli-Q water) and positive controls (DNA from plasmid pPDL11) were included in the PCR reaction. PCR products were analysed by agarose gel electrophoresis on a 2% gel stained with ethidium bromide (0.5 μg ml) in order to visualise the DNA bands.

3.2.10 Loss of Degrading Ability

The five oxamyl-degraders isolated from soil OX 5 were used in this experiment. To confirm that the isolates still retained their oxamyl-degrading ability, one bead was taken from each Protect Vial (–80°C storage), inoculated directly into 20 ml MSMN+OX (15 mg l^{-1}) and incubated at 25°C until more than 50% of the oxamyl had degraded, approximately 4-6 days. Sub-samples were taken periodically and analysed by HPLC to determine the oxamyl concentration. After incubation, a loop-full of culture was taken from each MSMN+OX culture and streaked on to solid MSMN+OX medium. Agar plates were incubated at 25°C for approximately 6 days. For each of the five strains, a well-isolated colony was picked off the MSMN+OX plates and inoculated into 20 ml Nutrient Broth (Oxoid). All were tested in duplicate and incubated at 25°C for 48 h. After incubation, 0.5 ml of each Nutrient Broth (NB) culture was transferred to 20 ml of fresh NB and incubated

for a further 48 h at 25°C. At the end of the second NB cycle, the cultures were serially diluted 10-fold by transferring 1 ml of culture into 9 ml of 0.85 % NaCl solution and 0.1 ml of each dilution was inoculated on to NA plates. All agar plates were incubated at 25°C for 4 days.

3.2.10.1 Test of Ability to Degrade Oxamyl

To test isolates for loss of degrading ability, 100 individual colonies per isolate were picked off the highest NA dilution plates showing growth and inoculated into microtitre plate wells. Each well contained 200 μl of MSMN+OX (15 mg l^{-1}). Ten wells were left uninoculated to act as abiotic controls and all microtitre plates were incubated at 25°C for 20 days. Immediately after inoculation of the test wells, a 100 μl sub-sample was taken from the 10 uninoculated control wells to gain an average value for the initial oxamyl concentration. The sub-sample was diluted with 200μl of methanol before analysis by HPLC (Section 3.2.2.1). Microtitre plates were sealed inside plastic bags to prevent loss by evaporation and not sampled again until day 20, following the above sampling procedure.

Loss of degrading ability from soil OX5 isolates was also attempted using elevated temperatures, an established curing method (Trevors 1986). Using colonies from MSMN+OX dilution plates (see above), well-isolated colonies were inoculated into 20 ml NB and incubated at 42°C for 48 h. Unfortunately soil OX5 isolates failed to grow in NB at this elevated temperature.

3.3 RESULTS

3.3.1 Enrichment Culture Preliminary Experiments

3.3.1.1 Media Test

Results from the preliminary investigation into the effects of different media supplements on oxamyl degradation by soil OX 10 demonstrated that Mineral Salts Media supplemented with a nitrogen source (MSMN) was the only media type in which the rate of oxamyl degradation was greater than that of the uninoculated control (Figure 11). This media was therefore used in all further enrichment cultures. Although it was later found that the 50:50 methanol and water oxamyl stock solution provided an additional carbon source in the form of methanol, the media test experiment was not repeated.

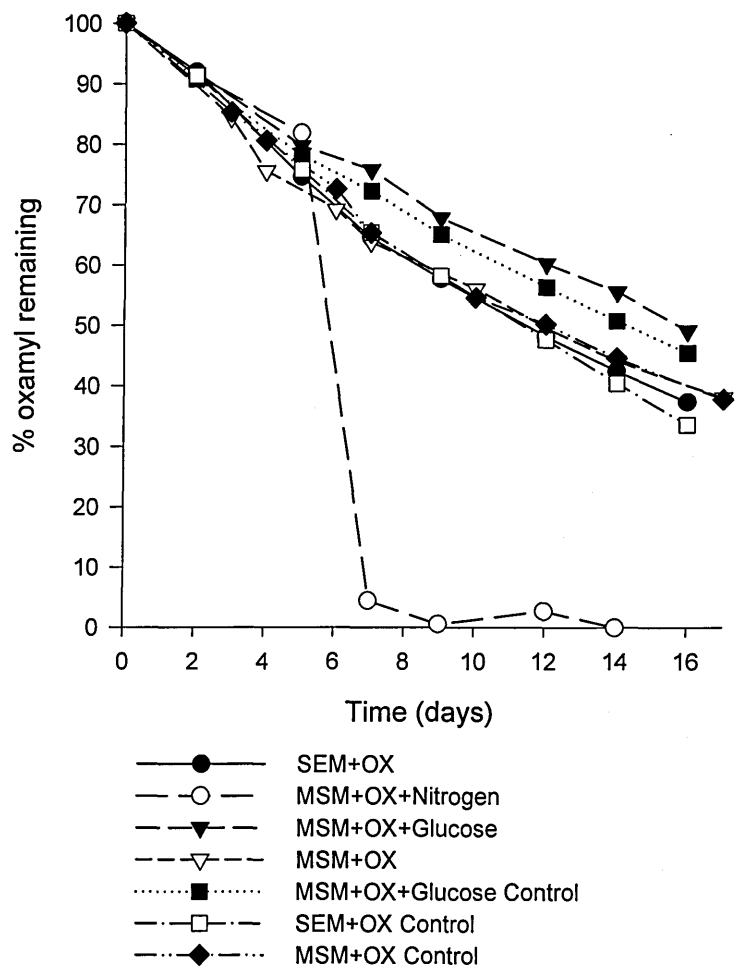


Figure 11: Degradation of oxamyl in media containing different nutritional supplements. All results are the mean (n=3) presented as a percentage of the day 0 concentration.

3.3.1.2 Initial Enrichment Culture and Oxamyl-Degrader Isolation (methanol present in the media)

Using the original method in which methanol, added to the media as part of the oxamyl solution, was not removed from the media and thereby provided an additional carbon source, rapid rates of oxamyl degradation were still observed in the three soils tested (OX4, OX7 and OX10). Results, however, are not shown because these experiments were later repeated using the methanol elimination method. An attempt to isolate oxamyl-degrading bacteria from soil OX10 yielded only one positively degrading isolate that could not be purified and which later lost its ability to degrade oxamyl. It was however, of use in the preliminary investigation, 'Does Methanol Provide an Additional Carbon Source in the Media?' (Section 3.2.5)

3.3.1.3 Methanol Elimination Experiment

Bacterial growth was determined by a visual inspection of the turbidity of the cultures. Media types one (oxamyl and methanol) and two (methanol only) became very turbid after 6 days. However, media type three (no oxamyl or methanol) remained as clear as the uninoculated control (media type four), demonstrating that the methanol, added to the media as part of the oxamyl solution, was providing an extra carbon source and so giving rise to the large number of non-oxamyl degrading bacteria.

3.3.2 Enrichment Culture Final Method

Using the modified method which eliminated methanol from the enrichment cultures resulted in less turbid growth, possibly because the vast majority of non-oxamyl degrading bacteria had been eliminated and also because of reduced growth of oxamyl-degraders in this now more nutritionally poor media. All soils tested in enrichment, except soil OX 11, demonstrated degradation rates faster than the uninoculated control and in all soils except OX10 oxamyl was degraded faster in the second and third enrichment cycles than in the

first (Figure 12). Soil OX 11 had previously failed to demonstrate enhanced rates of degradation in the soil incubation study (Chapter 2). All soils that demonstrated rapid degradation in the enrichment culture were taken on to attempt to isolate oxamyl-degrading bacteria.

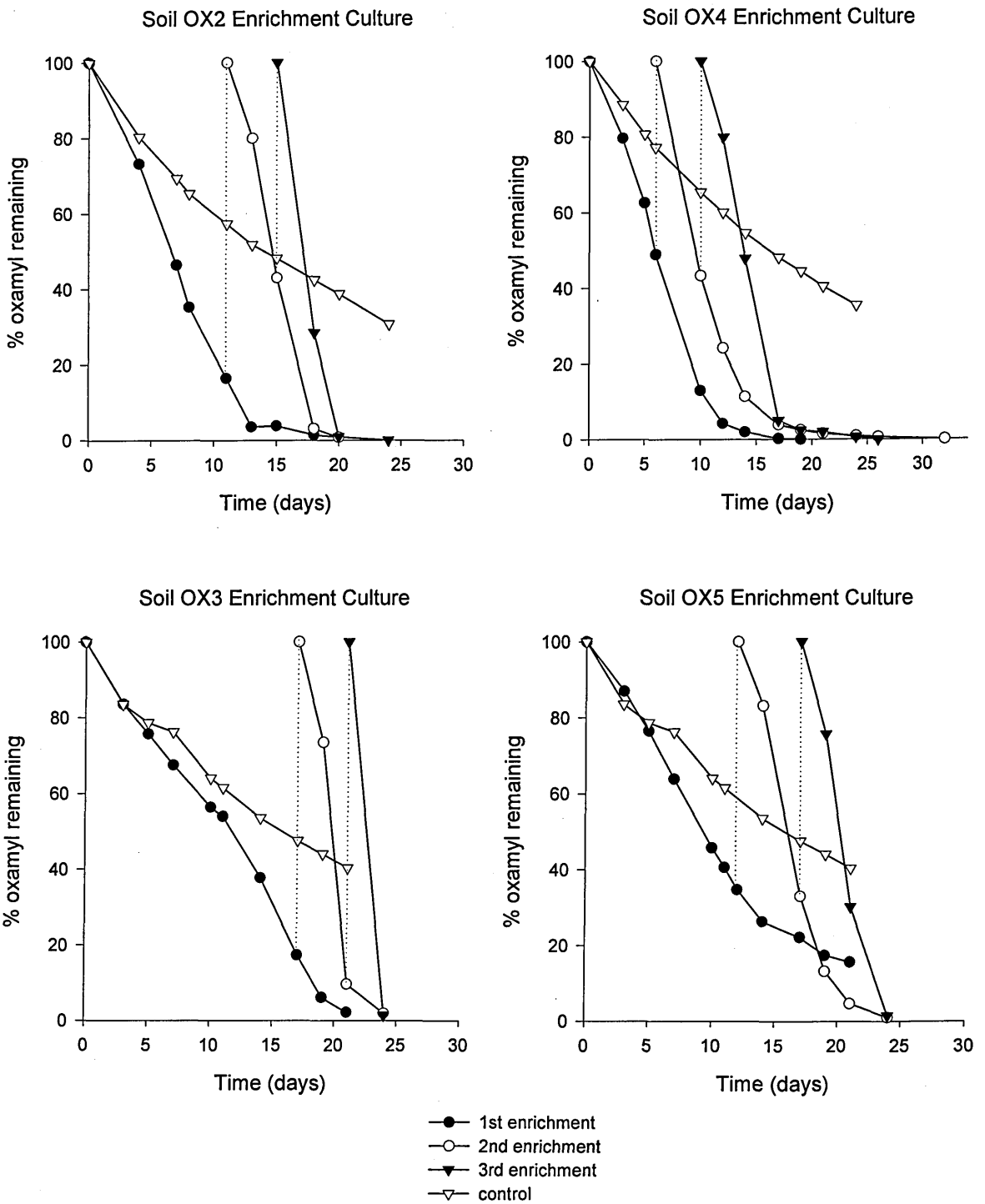


Figure 12: Degradation of oxamyl in enrichment culture by different soils. All results are the means (n=3) presented as a percentage of the day 0 concentration. Vertical dotted lines indicate the point at which 0.5 ml of culture was transferred to fresh MSMN+OX media.

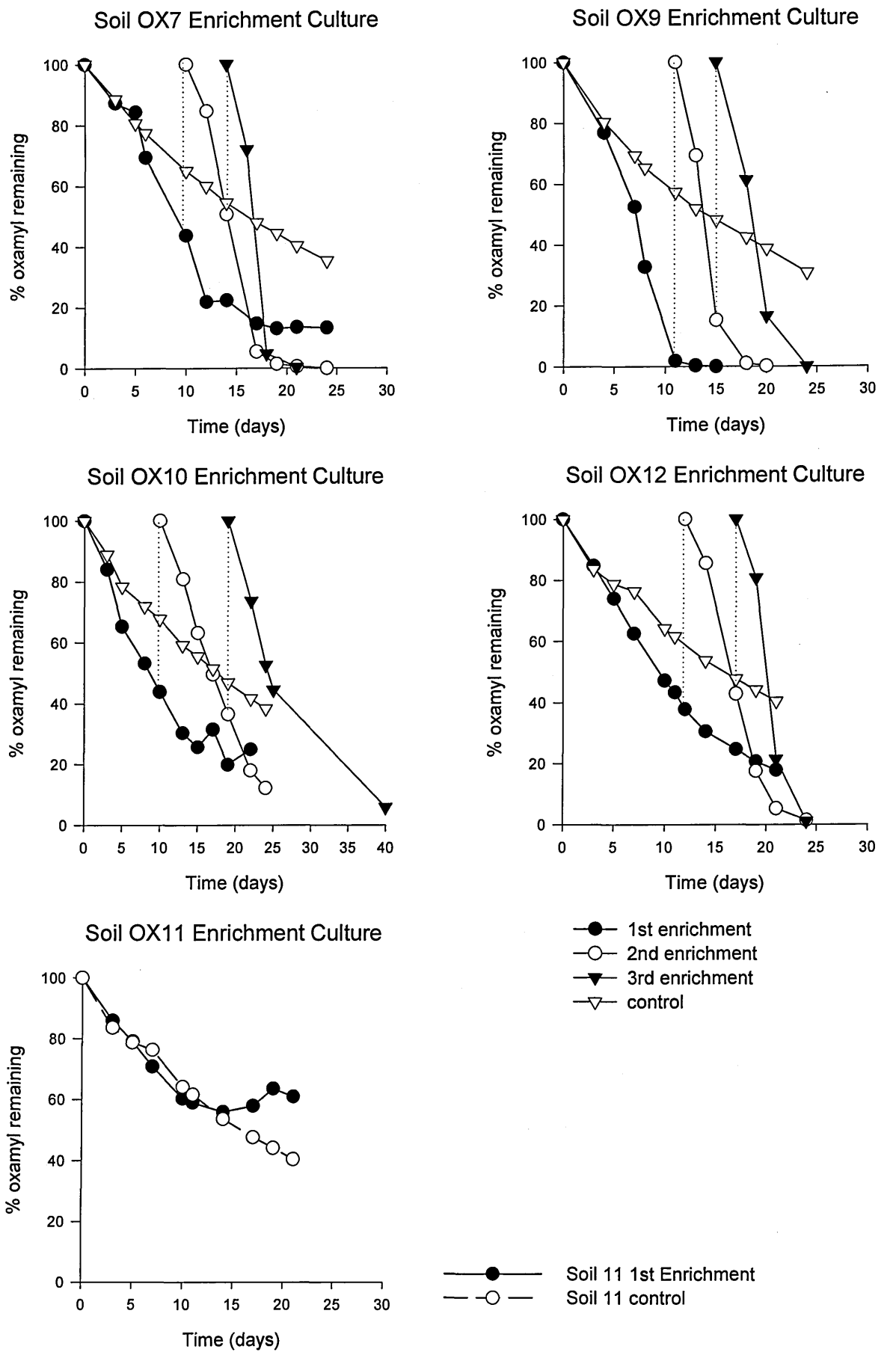


Figure 12 continued: Degradation of oxamyl in enrichment culture by different soils. All results are the means (n=3) presented as a percentage of the day 0 concentration. Vertical dotted lines indicate the point at which 0.5 ml of culture was transferred to fresh MSMN+OX media.

3.3.3 Isolation of Oxamyl-Degrading Bacteria

All rapidly degrading third-cycle enrichment cultures successfully yielded colony types that demonstrated rapid oxamyl degradation. However, the number of rapidly degrading cultures varied between each soil, with some soils yielding a much higher number than others (Table 9). Individual degradation rates for all colonies tested in test culture are displayed in Chapter 7, Tables 7.4 to 7.11.

Table 9: Number of colonies displaying rapid oxamyl degradation in test cultures for each soil.

Soil	***Number of rapidly degrading test cultures***
OX2	7
OX3	4
OX4	11
OX5	5
OX7	2
OX9	5
OX10	6
OX12	3

In most cases, 30 or more colonies per soil were sub-cultured from the serial dilution plates of the third enrichment cycle; however, in reality not all of these colonies grew after sub-culturing, reducing the overall number of isolates taken on to be tested for oxamyl-degrading ability. Also, once found to degrade oxamyl not all isolates could be purified, further reducing the number that could ultimately be characterised. In total, rapid oxamyl degradation was observed in 43 cultures from 8 different soils; however, only 36 isolates were successfully identified as can be seen in Section 3.3.4.

3.3.4 Characterisation of Oxamyl-degrading Bacteria

3.3.4.1 Phenotypic Tests

All successfully purified isolates were found to be Gram-negative, aerobic, rod-shaped bacteria and all bar one were oxidase-positive and catalase-negative. The only successfully purified soil OX 12 isolate was found to be oxidase-negative and catalase-positive. A number of the soil OX 4 and OX 7 isolates were tested using API 20NE strips; however, in all cases these could not successfully identify the isolates. For this reason, and because of the high cost of the strips, use of this system was abandoned in favour of the more successful 16S rRNA gene amplification method. As the API system was not successful, the results are not displayed.

3.3.4.2 Partial Sequencing of the 16S rRNA Gene and Alignment

Of the initial 43 oxamyl-degrading cultures, 36 isolated strains were successfully identified; the remainder could not be purified or could not be sequenced once purified, including all of the soil OX 9 isolated strains. Results from sequencing and alignment of the 16S rRNA gene are displayed in Table 10. At the species level there appears to be little diversity amongst the isolates, which were isolated from agricultural soils originating from different geographical locations around England. The oxamyl-degrading strains showed similarity to only four species, which can be classified as: bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Rhizobiaceae, *Chelatobacter heintzii*; bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Phyllobacteriaceae, *Aminobacter aminovorans*; bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Phyllobacteriaceae, *Aminobacter niigataensis*; bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Phyllobacteriaceae, *Mesorhizobium sp.* The predominant genus was *Aminobacter*, which appeared in six of the seven soils, a total of 35 of the 36 isolates. The remaining isolate was identified as belonging to the genus *Mesorhizobium*. Twenty-one of

the 36 isolates demonstrated strong similarity to the species *Aminobacter aminovorans* which occurred in five of the seven soils from which isolate identification was successful. However, all soil OX 10 isolates and some from soils OX 3 and OX 2 demonstrated an equal degree of similarity to both *Aminobacter niigataensis* and *Chelatobacter heintzii.* Further tests would be required to determine which species the isolates most closely matched. The only isolate successfully identified from soil OX 12 gave a 98% match to *Mesorhizobium* sp., a genus only encountered in two of the other isolates, a soil OX 4 isolate and one from soil OX 5. However, these two isolated strains only yielded short readable sequences of 184 bp (soil OX5 isolate) and 162 bp (soil OX4 isolate) making any sequence alignment unreliable. In addition, these two isolates also showed an equal degree of similarity to *Aminobacter aminovorans.*

Of the 36 oxamyl-degrading strains isolated from 7 soils, 19 belonged to the genus *Aminobacter*, 14 demonstrated an equal degree of similarity to the genus *Aminobacter* and *Chelatobacter*, 2 demonstrated an equal degree of similarity to the genus *Aminobacter* and *Mesorhizobium*; and 1 belonged to the genus *Mesorhizobium.* No sequence differences were observed when 16S rRNA sequences of strains matching *A. aminovorans* were aligned and, similarly, there were no differences between strains matching both *A. niigataensis* and *C. heintzii.*

Within-soil variability was also low. The highest number of different species isolated from a soil was three. However, a number of the isolates from these soils, soils OX 2 and OX 3, bore an equal degree of similarity to more than one of the species, namely *Aminobacter niigataensis* and *Chelatobacter heintzi.*

Table 10: Partial 16S rRNA gene sequencing and alignment; and presence of the mcd *gene for oxamyl-degrading bacteria isolated from UK agricultural soils.*

Isolate origin	***Isolate code***	***Nearest neighbours***	***Accession number***	***% similarity***	***Size (bp)***	***mcd gene***
Soil OX7 Norfolk	7/6/A	*Aminobacter aminovorans*	AF329835	100	354	-
	73S3/2	*Aminobacter aminovorans*	AF329835	100	469	-
Soil OX5 Norfolk	52S3/10	*Mesorhizobium chacoense*	AJ278249	99	184	ND
		Aminobacter aminovorans	AF329835	99		
	52S3/9	*Aminobacter aminovorans*	AF329835	100	282	ND
	52S3/2	*Aminobacter aminovorans*	AF329835	99	271	ND
	52S3/8	*Aminobacter aminovorans*	AF329835	99	517	-
	51S3/6	*Aminobacter aminovorans*	AF329835	99	569	-
Soil OX4 Lincolnshire	41S3/5	*Mesorhizobium chacoense*	AJ278249	98	162	ND
		Aminobacter aminovorans	AF329835	98		
	42S3/7	*Aminobacter aminovorans*	AF329835	100	309	ND
	42S3/7A	*Aminobacter aminovorans*	AF329835	99	369	-
	42S3/1	*Aminobacter aminovorans*	AF329835	99	518	-
	42S3/1A	*Aminobacter aminovorans*	AF329835	100	411	ND
	42S3/3	*Aminobacter aminovorans*	AF329835	100	469	ND
	42S3/3A	*Aminobacter aminovorans*	AF329835	99	507	ND
	42S3/4	*Aminobacter aminovorans*	AF329835	99	509	-
	42S3/3B	*Aminobacter aminovorans*	AF329835	99	431	ND
	42S3/5	*Aminobacter aminovorans*	AF329835	99	532	-
Soil OX3 Lincolnshire	32S3/10	*Aminobacter niigataensis*	AJ011761	100	609	-
		Chelatobacter heintzii	AJ011762	100		
	31S3/8	*Aminobacter aminovorans*	AF329835	100	385	-
Soil OX2 Lincolnshire	23S3/2A	*Aminobacter niigataensis*	AJ011761	99	299	(+)
		Chelatobacter heintzii	AJ011762	99		
	23S3/1	*Aminobacter niigataensis*	AJ011761	100	510	(+)
		Chelatobacter heintzii	AJ011762	100		
	23S3/1A	*Aminobacter niigataensis*	AJ011761	100	449	(+)
		Chelatobacter heintzii	AJ011762	100		
	23S3/2	*Aminobacter aminovorans*	AF329835	99	663	-
	21S3/1	*Aminobacter aminovorans*	AF329835	99	648	-
	21S3/2	*Aminobacter aminovorans*	AF329835	100	312	-
	22S3/4	*Aminobacter niigataensis*	AJ011761	100	599	(+)
		Chelatobacter heintzii	AJ011762	100		
	23S3/3	Sequencing unsuccessful				(+)

Results for PCR amplification of sequences homologous to the *mcd* gene are for primer set two only
(+)- *mcd* gene sized bands present but only faintly visible
ND- presence of the *mcd* gene was not determined using primer set two

Table 10 continued: Partial 16S rRNA gene sequencing and alignment; and presence of the mcd gene for oxamyl-degrading bacteria isolated from UK agricultural soils.

Isolate origin	*Isolate code*	*Nearest neighbours*	*Accession number*	*% similarity*	*Size (bp)*	*mcd gene*
Soil OX12 Hampshire	121S3/9	*Mesorhizobium* sp.	AF156710	98	540	-
Soil OX10 Cornwall	102S3/12	*Aminobacter niigataensis*	AJ011761	99	300	-
		Chelatobacter heintzii	AJ011762	99		
	102S3/13A	*Aminobacter niigataensis*	AJ011761	100	337	-
		Chelatobacter heintzii	AJ011762	100		
	102S3/13	*Aminobacter niigataensis*	AJ011761	100	418	(+)
		Chelatobacter heintzii	AJ011762	100		
	102S3/3	*Aminobacter niigataensis*	AJ011761	99	697	(+)
		Chelatobacter heintzii	AJ011762	99		
	101S3/15A	*Aminobacter niigataensis*	AJ011761	100	386	-
		Chelatobacter heintzii	AJ011762	100		
	101S3/15	*Aminobacter niigataensis*	AJ011761	99	376	-
		Chelatobacter heintzii	AJ011762	99		
	101S3/2	*Aminobacter niigataensis*	AJ011761	100	665	(+)
		Chelatobacter heintzii	AJ011762	100		
	102S3/1	*Aminobacter niigataensis*	AJ011761	99	349	(+)
		Chelatobacter heintzii	AJ011762	99		
	101S3/9	*Aminobacter niigataensis*	AJ011761	100	316	(+)
		Chelatobacter heintzii	AJ011762	100		

Results for PCR amplification of sequences homologous to the *mcd* gene are for primer set two only
(+)- *mcd* gene sized bands present but only faintly visible
ND- presence of the *mcd* gene was not determined using primer set two

3.3.5 PCR Detection of the Mcd Gene

Successfully characterised oxamyl-degrading strains were assessed for *mcd*-gene sequence homology using the PCR detection method. Two different primer sets were used to try to improve the chances of detecting homologous sequences: Primer set one (Parekh *et al.*, 1996), designed to amplify a 561 bp fragment and primer set two (Derk *et al.*, 2003), designed to amplify a 406 bp fragment. Results for PCR detection of sequences homologous to the *mcd* gene using primer set two are displayed in Table 10. All characterised strains, plus one other Soil OX 2 isolate that could not be sequenced, were tested for *mcd*-gene homologous sequences using Primer set one. However, no *mcd* gene-

sized bands were visualised under gel electrophoresis (results not shown). Results with primer set two were, however, more successful as can be seen from Figures 13 and 14.

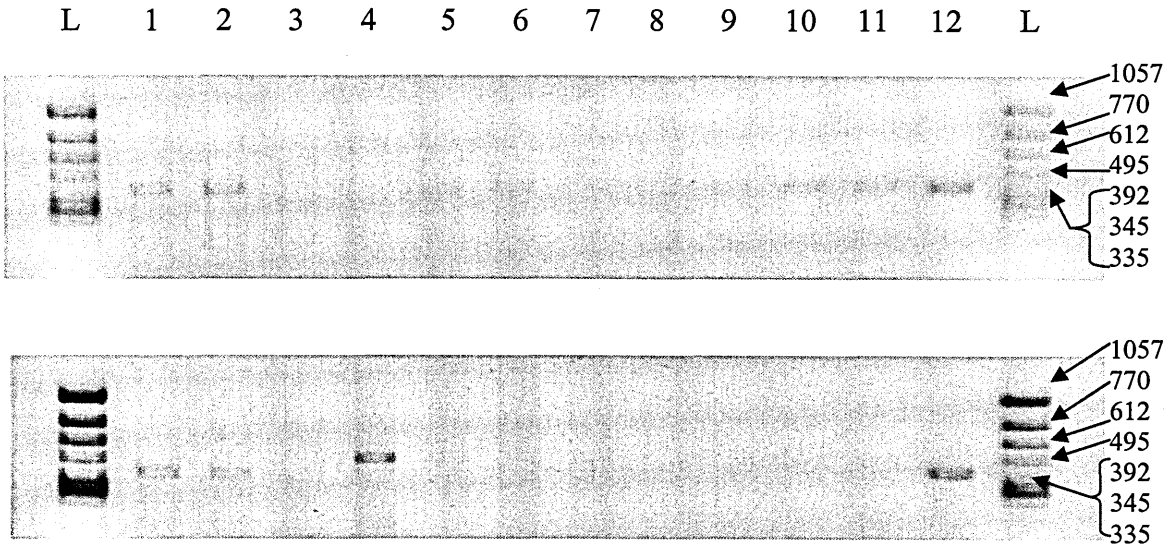


Figure 13: Agarose gel showing PCR products obtained from oxamyl-degrading bacteria using primer set two (Derk et al., 2003). L, molecular weight ladder (ØX174 Hinc II) in base pairs; lanes1-12 (top gel): 23S3/1A, 23S3/1, 23S3/2, 21S3/1, 22S3/4, 102S3/13, 102S3/13A, 101S3/15A, 101S3/15, 102S3/1,101S3/9, pPDL11 (positive control). Lanes 1-12 (bottom gel): 101S3/2, 102S3/3, 32S3/10, 51S3/6, 52S3/8, 42S3/1, 42S3/5, 73S3/2, 42S3/7A, 42S3/4, 7/6A, pPDL11 (positive control).

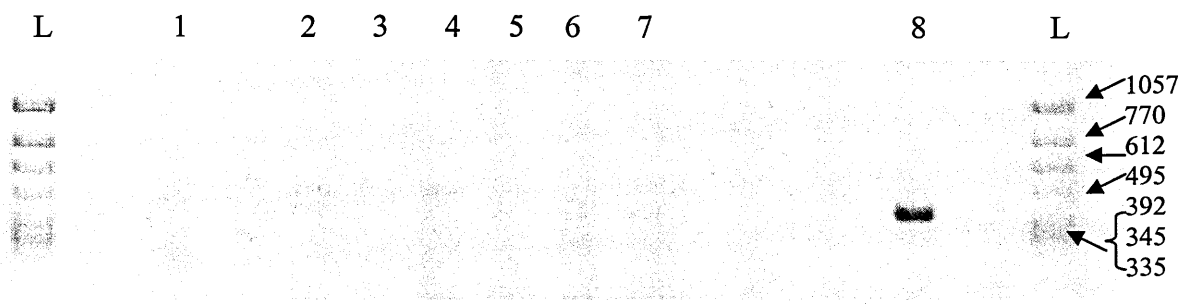


Figure 14: Agarose gel showing PCR products obtained from oxamyl-degrading bacteria using primer set two (Derk et al., 2003). L, molecular weight ladder (ØX174 Hinc II) in base pairs; lanes 1-8: 121S3/9, 21S3/2, 73S3/2, 31S3/8, 102S3/12, 23S3/3, 23S3/2A, pPDL11 (positive control).

Out of the 28 isolated strains tested using primer set two, ten displayed faintly visible bands of the same molecular weight as the *mcd* gene positive control (DNA from plasmid pPDL11 isolated from *Achromobacter* sp. strain WM111). A strong band was detected in an isolate from soil OX 5; however this was of a larger molecular weight than the *mcd*

gene positive control. All *mcd* positive strains were isolated from the geographically distinct soils OX 2 and OX 10. None of the successfully characterised strains isolated from the remaining five soils were found to contain the *mcd* gene, although not all of the soil OX 4 and OX 5 isolates were tested with primer set two. Alignment results of 16S rRNA gene partial sequences from *mcd* gene positive isolates show that all of these isolates demonstrated an equal degree of similarity to the species *Aminobacter niigataensis* and *Chelatobacter heintzii*.

3.3.6 Loss of Oxamyl-Degrading Ability

A number of studies have attempted plasmid curing by growing bacteria in the absence of the pesticide that the bacteria utilises as a nitrogen or carbon source (Head *et al.*, 1992; Feng *et al.*, 1997; Turnball *et al.*, 2001). These studies frequently report loss of degrading ability after successive sub-culturing in liquid media that does not contain the target pesticide. Easily lost degrading ability can imply the possible involvement of plasmids in degradation. It was observed that the oxamyl-degrading isolates did not degrade oxamyl if taken directly from –80°C storage and cultured in the absence of oxamyl. As a result of this observation, oxamyl-degrading strains isolated from soil OX 5 were successively cultured in the absence of oxamyl in an attempt to rid them of their degrading ability. Use of the elevated temperature method to produce strains unable to degrade oxamyl failed, as none of the OX5 isolates tested grew at 42°C. Results at 25°C were more successful and are displayed in Table 11.

Table 11: Percentage loss of degrading ability after successive sub-culturing of oxamyl-degrading bacteria in the absence of oxamyl

Isolate origin	***Isolate Code***	***% of colonies that lost oxamyl-degrading ability***
Soil OX5 Norfolk	51S3/6	1
	52S3/2	0
	52S3/8	47
	52S3/9	2
	52S3/10	1

Of the five strains tested, only one demonstrated a notable loss of degrading ability with 47 of the 100 colonies losing oxamyl-degrading ability. Loss from the remaining four strains was negligible.

3.4 DISCUSSION

3.4.1 Enrichment Culture Studies

Inoculation of UK agricultural soils, that had previously demonstrated enhanced degradation in a soil incubation study (Chapter 2), into mineral salts medium that contained oxamyl as the sole carbon source resulted in the rapid disappearance of oxamyl. The persistence of oxamyl decreased with successive sub-culture. Rapid degradation of oxamyl in the second and third cultures took place in the absence of soil and with oxamyl as the sole carbon source. The rate of oxamyl degradation in the first enrichment for a number of the soils, particularly soils OX 3, 7 and 9, was initially slower, indicating a possible lag phase, prior to a more rapid rate of degradation which tailed off as concentrations became too low to support growth (Figure 12). It is thought that during the lag phase, soil microorganisms are adapting in some way and an increase in the size of the population capable of metabolising the chemical results in the subsequent faster rate of biodegradation (Kearney and Kellogg, 1985). This phenomenon has been observed with a number of pesticide-degrading microorganisms (Charney and Fournier, 1994; Trabue *et al.*, 2001; Bending *et al.*, 2003; Sorensen and Aamand, 2003).

Also of interest is the lack of rapid oxamyl degradation by soil OX 11 (Figure 12). Degradation of oxamyl by soil OX 11 in the soil incubation study (Chapter 2) was also slow in comparison to the other soils tested, suggesting that soil microorganisms adapted to degrade oxamyl are not present in this soil. These findings could suggest that the

enrichment culture method is representative of the occurrence of enhanced degradation in laboratory incubation studies and thus of enhanced degradation in the field (Chapter 4). Testing this hypothesis with a number of different soils would, of course, be necessary as Rousseaux *et al.* (2001) have already reported the successful enrichment and isolation of atrazine-degrading bacteria from soils that had previously only degraded the pesticide at a slow rate in soil incubation.

3.4.2 Isolation and Identification of Oxamyl-Degrading Bacteria

Colonies capable of rapid oxamyl degradation in liquid medium were successfully isolated from third-cycle enrichment cultures for all rapidly degrading soils tested. However, not all of these colonies could be purified or sequenced. Ultimately 36 oxamyl-degrading strains, isolated from seven soils taken from six different farms across England, were characterised. All oxamyl-degrading strains were Gram-negative, aerobic rod-shaped bacteria. At the species level, there appears to be little diversity amongst the isolated strains, with 36 isolates from seven different soils showing similarity to only four different species: *Aminobacter aminovorans, Aminobacter niigataensis, Chelatobacter heintzi* and *Mesorhizobium chacoense*. Thirty-five of the 36 (six of the seven soils) isolated strains demonstrated strong similarity to the genus *Aminobacter,* and 21 of these 36 strains aligned well (>98% similarity) to a 16S rRNA gene sequence from the species *Aminobacter aminovorans*. Out of the remaining isolates, 14 demonstrated similarity to both *Aminobacter niigataensis* and *Chelatobacter heintzii* and one to *Mesorhizobium* sp. Two isolates also demonstrated a high degree of similarity to both *A. aminovorans* and *Mesorhizobium chacoense*; however, only very short 16S rRNA sequences were obtained for these isolates rendering any alignment results unreliable. Oxamyl-degraders that demonstrated an equal degree of similarity to both *Aminobacter niigataensis* and *Chelatobacter heintzii* strains were isolated from three of the seven soils. Further work would be required to better establish which species these isolates can be assigned to. The

strong generic relationship between members of the *Aminobacter* genus and the *Chelatobacter* genus has been established, and it has been suggested that *Chelatobacter heintzii* is so similar to *A. aminovorans* that it is in fact a synonym of *A. aminovorans* (Kampfer *et al*, 2002). This further demonstrates the similarity amongst the oxamyl-degrading strains at the species level.

This appears to be the first report of strains belonging to the species *A. aminovorans, C. heintzii, A. niigataensis* and the genus *Mesorhizobium* that are capable of degrading oxamyl. *Aminobacter aminovorans, A. niigataenisis* and *Chelatobacter heintzii* are ubiquitous in the environment and strains of these species have previously been isolated from enhanced pesticide-degrading soils. *Aminobacter aminovorans* strains capable of mineralizing the herbicide atrazine (Rousseaux *et al.*, 2001) and the carbamate insecticide carbofuran (Desaint *et al.*, 2000) have been reported. Rousseaux *et al.* (2001) reported the isolation of *Chelatobacter heintzii* strains capable of atrazine degradation. Carbofuran-degrading *Aminobacter niigataensis* strains have also been described previously (Desaint *et al.*, 2000). The single isolate successfully purified and identified from Soil OX 12 gave a 98% similarity to *Mesorhizobium* sp. Pesticide-degrading *Mesorhizobium* sp. strains isolated from enhanced degrading soils has not been reported previously. A *Mesorhizobium* sp. capable of degrading copper-nitrilotriacetate complexes for the purpose of bioremediation has been reported (White and Knowles, 2003).

The 36 oxamyl-degrading strains could be assigned to only three genera, *Chelatobacter*, *Aminobacter* and *Mesorhizobium*. This lack of diversity amongst isolates from different soils and also within the same soil is in contrast to previous studies investigating the degradation of other methylcarbamate nematicides-insecticides. These studies found a high degree of genotypic diversity amongst carbofuran-degraders (Parekh *et al.*, 1995; Desaint *et al.*, 2000). These two studies did, however, investigate the relatedness amongst isolates

in greater detail. A number of different molecular techniques were employed, including amplified ribosomal DNA restriction analysis of the 16S rRNA gene and restriction fragment length polymorphism analysis (RFLP) (Parekh *et al.*, 1995; Desaint *et al.*, 2000). This allowed the isolates to be grouped by ribotype as well as by species. Desaint *et al.* (2000) reported isolates that were designated as the same species based on 16S rRNA sequences but belonged to different ribotype groups, and were therefore genetically different. Similarly, Parekh *et al.* (1995) found that phenotypically similar carbofuran-degrading isolates displayed different RFLP patterns and as such these isolates were not genetically identical. More in depth molecular analysis of the oxamyl-degrading strains reported in this study might demonstrate a degree of genetic diversity amongst isolates assigned to the same species.

This study is not alone in failing to report a high degree of diversity amongst pesticide-degrading isolates. Rousseaux *et al.* (2001) reported the isolation of 26 atrazine-degrading strains from ten different French soils that could be assigned to only four different species. Twenty of these isolates were assigned to one species, *Chelatobacter heintzii.* Similarly, Chaudhry and Ali (1988) and Karpouzas *et al.* (2000a) failed to report a high degree of diversity amongst their carbofuran-degrading isolates. The majority of isolates were designated as *Pseudomonas* strains. It is suspected that the enrichment culture method selects for fast-growing, culturable organisms or those adapted to the complex media used in the enrichment studies and may not be truly representative of the pesticide-degrading microbial community within the soil (Karpouzas *et al.*, 2000a). Rousseax *et al.* (2001) postulated that the enrichment method used might have introduced a bias resulting in the lack of diversity observed amongst their atrazine-degrading strains, which may not be truly representative of the atrazine-degrading community in the soil. The enrichment method used by Desaint *et al.* (2000), who reported a high degree of diversity amongst their carbofuran-degrading strains, was more varied as some enrichment cultures received

higher concentrations of carbofuran and some were treated with aldicarb. Although this method appears to result in the isolation of a more diverse range of carbofuran-degrading strains, it does limit between-soil comparisons.

Thirty-five of the oxamyl-degrading strains isolated from six different soils showed a high degree of similarity to species from the *Aminobacter* genus. This lack of species diversity amongst and within soils provides support for the idea of using specific bacterial species as indicators of a soil's potential for enhanced oxamyl degradation. A number of questions do, however, have to be asked. Have all major species involved in oxamyl-degradation been isolated? Do these isolated strains actually degrade oxamyl within the soil? Were these isolates constructed during the repeated sub-culturing involved in the isolation procedure? As mentioned above, the enrichment method used may not necessarily result in the isolation of strains that are truly representative of the degrading community within the soil. Also, a large proportion of the soil microbial population is known to be unculturable (Marchesi *et al.*, 1998) so it is unlikely that all the major oxamyl-degrading species have been isolated. Evidence for the involvement of isolated pesticide-degrading strains in the actual degradation of the chemical within the soil is available (Bending *et al.*, 2003; Singh *et al.*, 2003b), however, further testing would be required to establish whether this was true for the oxamyl-degrading strains reported in this thesis. In response to the final question, there is evidence to suggest that the repeated sub-culturing involved in the enrichment method can lead to genetic changes in the bacteria capable of degrading the chemical (Fernandez *et al.*, 1999; Newby *et al.*, 2000; Bending *et al.*, 2003; Desaint *et al.*, 2003). This would come about as a result of gene transfer between bacteria in the enrichment culture, conveying oxamyl-degrading ability upon strains that did not previously degrade oxamyl. Isolated oxamyl-degrading strains could therefore have been constructed during the isolation process and not actually be present as such in the soil (personal communication from Guy Soulas, 2003).

It should also be noted that although the alignment of partial sequences of the 16S rRNA gene from oxamyl-degrading strains has given good similarity to strains on the GenBank database, partial sequencing is often insufficient for precise strain identification (Desaint *et al.*, 2000). This is particularly so as some of the isolates had very short partial sequences (184 bp, soil OX 5 isolate and 162 bp, soil OX 4 isolate). Full sequencing of this gene would allow more accurate identification. It would be of particular use with those isolates that demonstrated an equal degree of similarity to more than one species. In addition, the sequence alignment results are only as good as the sequences available on the database. New sequences are continually being added to the database and as such re-running the sequence alignments at a later date may result in closer matches to sequences that were not previously available on the database.

3.4.3 PCR Detection of the Mcd *Gene*

Two different primer sets were used in this experiment, but one set (Parekh *et al.*, 1996) failed to detect *mcd* gene homologous sequences in any of the isolates tested. The second primer set (Derk *et al.*, 2003) gave faint bands of a similar molecular weight to the *mcd* gene in ten of the 28 isolates that were screened with these primers. In addition, a strong band, larger than the *mcd* gene, was detected in an isolate from soil OX 5. In order to determine whether this larger band is homologous to the *mcd* gene, the PCR product would need to be sequenced and aligned with the *mcd* gene sequence. Not all characterised oxamyl-degrading strains were screened using this primer set because the primers were not obtained until close to the end of the three-year project. Isolates displaying faint bands of a similar size to the *mcd* gene were from only two of the seven soils, soils OX 2 and OX 10 originating from fields in Lincolnshire and Cornwall respectively. All *mcd* gene positive isolates showed a high degree of similarity to both *Aminobacter niigataensis* and *Chelatobacter heintzii*. These findings are supported by other studies that also failed to detect the *mcd* gene in all methylcarbamate-degrading strains (Parekh *et al.*, 1995; Parekh

et al., 1996; Hashimoto *et al.*, 2002). Desaint *et al.* (2000) screened 128 carbofuran-degrading bacteria for *mcd* gene homologous sequences using the same primer sequences as primer set one (Parekh *et al.*, 1996). They detected the *mcd* gene in only 58 of the isolates. As with the oxamyl-degrading strains reported in this thesis, Desaint *et al.* (2000) also isolated *mcd* gene positive and *mcd* gene negative strains from the same soil.

To ensure that a large amount of DNA could be extracted for use in the PCR detection procedure, oxamyl-degrading isolates were cultured in mineral salts media that contained an extra nitrogen and carbon source in addition to oxamyl. The potential for any gene involved in oxamyl degradation to be encoded on a plasmid and thus easily lost if the selective pressure of a nutritionally poor environment was removed was taken into consideration. The degradation rate of oxamyl was monitored in all cultures to ensure rapid degradation was occurring. The *mcd* gene had therefore not been lost (if it had ever been present) as a result of culturing the isolate in a medium containing glucose as an extra carbon source in addition to oxamyl. It can therefore be assumed that *mcd* gene negative isolates are as a result of failing to detect sequences homologous to the *mcd* gene using *mcd* gene specific primers and are not a result of the culturing method. The failure of primer set one to detect the *mcd* gene in any of the isolates may suggest that the primer sites cover an area of sequence that is less highly conserved than that covered by primer set two. An alternative *mcd* gene detection method would be to use an *mcd* gene probe cloned from plasmid DNA containing the *mcd* gene sequence. The probe would hybridise with similar gene sequences, thus indicating the presence of the *mcd* gene (Topp *et al.*, 1993).
The *mcd* gene is not specific to carbofuran degradation and has been shown to be involved in the degradation of other methylcarbamates (Derbyshire *et al.*, 1987; Topp *et al.*, 1993). As such it was hypothesised that the *mcd* gene might be present in the oxamyl-degrading strains isolated in this study. There does appear to be evidence for the presence of the *mcd* gene in oxamyl-degrading strains isolated from two geographically different soils. Of

course, further work, such as sequencing the DNA from the bands and aligning with the *mcd* gene sequence, is required to confirm whether the faint bands that were visualised under gel electrophoresis do contain DNA sequences homologous to the *mcd* gene. The results from this study do not, however, provide supporting evidence for the use of the *mcd* gene as an indicator of a soil's potential to develop enhanced oxamyl degradation because the majority of isolates were *mcd* gene negative and both *mcd* gene positive and negative strains were isolated from the same soil.

3.4.4 Loss of Oxamyl-Degrading Ability

Genes involved in the enhanced degradation of pesticides by microorganisms have frequently been found to be encoded on plasmids (Serdar *et al.*, 1982; Mulbry *et al.*, 1986; Tomasek and Karns, 1989; Topp *et al.*, 1993; Feng *et al.*, 1997; Zhongli *et al.*, 2001; Hashimoto *et al.*, 2002), a strategy that affords the organisms an evolutionary advantage (Kearney and Kellogg 1985; Karns, 1990). As the *mcd* gene was not detected in all oxamyl-degrading isolates tested, it was thought that it might be possible to at least indicate whether or not the gene encoding oxamyl hydrolysis was located on a plasmid. However, only one of the five soil OX 5 isolates tested demonstrated a notable loss of degrading ability, with 47% of cells unable to degrade oxamyl after repeated sub-culture in the absence of the nematicide. Further work, such as plasmid DNA profiling and purification, would be required to confirm whether the plasmid composition of potentially cured strains is different from that of degrading strains.

The method used to attempt to cure soil OX 5 isolates relied upon observations made whilst working with oxamyl-degrading isolates in the laboratory. It had been noted that when isolates were taken from –80°C storage and cultured in NB in the absence of oxamyl, they failed to degrade oxamyl when sub-cultured into mineral salts media containing oxamyl as the sole carbon source. Previous studies have reported success with similar

plasmid-curing methods, where the isolate is grown in the absence of the pesticide (Feng *et al.*, 1997; Turnball *et al.*, 2001). Head *et al.* (1992) grew their carbofuran-degrading strain in mineral salts medium containing yeast and peptone but no carbofuran at 25°C for 48-60 h, resulting in 70% of cells losing the ability to completely degrade carbofuran. By analysing the plasmid content of degrading strains and cured strains they concluded that one plasmid (designated pIH3) was always present in strains capable of totally degrading carbofuran and always absent from cured strains unable to degrade carbofuran phenol. However, it is likely that the method reported here requires further development and therefore the results with the five soil OX 5 isolates can not be relied upon. Certainly, if the remaining oxamyl-degrading strains from soils OX 2, 3, 4, 7, 9, 10 and 12 are to be cured by sub-culture in the absence of oxamyl then this method will first require some modification.

One possible modification to the curing method would be to increase the number of times the cultures are sub-cultured in NB. This may improve loss rate. Turnball *et al.* (2001) sub-cultured their diuron-degrader in media, that did not contain diuron, every 24 h for 10 days. Examining the plasmid profiles of cured strains and strains still able to degrade diuron revealed that cured strains were lacking a plasmid that was present in the degrading strains. Feng *et al.* (1997) grew their carbofuran-degrading bacteria in the absence of carbofuran but at the elevated temperature of 42°C. This method was attempted with the soil OX 5 isolates; however they failed to grow at this temperature and the method was abandoned. Another option is the use of chemicals to remove plasmids. Mitomycin C, rifampicin, and ethidium bromide are a few of the chemicals frequently and successfully used to cure plasmids (Head *et al.*, 1992; Hayatsu *et al.*, 1999; Deshpande *et al.*, 2001). These chemicals do however pose health and safety issues, the main reason they were not used in this study.

3.4.5 Conclusions

- All soils that demonstrated enhanced degradation in the soil incubation study (Chapter 2) also showed rapid degradation of oxamyl in enrichment culture. Soil OX 11 that degraded oxamyl at a relatively slow rate during soil incubation also failed to demonstrate rapid oxamyl degradation when tested in enrichment culture.

- Oxamyl-degrading bacteria were isolated from the third enrichment culture cycle for all soils that demonstrated enhanced degradation in soil incubation, although not all isolates could be purified or characterised.

- A high diversity amongst oxamyl-degrading isolates was not observed. The 36 strains isolated from seven geographically different soils showed similarity to only four different species: *Aminobacter aminovorans, Aminobacter niigataensis, Chelatobacter heintzi* and *Mesorhizobium chacoense*. Twenty-one of these isolates displayed a strong similarity to *A. aminovorans*.

- Using the PCR detection method to detect *mcd* gene homologous sequences resulted in the visualization of faint bands of a similar molecular weight to the *mcd* gene in ten of the 28 isolates examined. Further work is required to confirm whether these bands do contain *mcd* gene homologous sequences

- Sub-culturing oxamyl-degrading isolates in the absence of oxamyl was largely unsuccessful as only one of five isolates tested demonstrated a notable of loss of degrading ability. Further development of the method is required.

4. INVESTIGATING IDEAS FOR A DIAGNOSTIC ASSAY OF NEMATICIDE PERSISTENCE IN SOILS

4.1 INTRODUCTION

Granular nematicides are one of the main tools available to UK farmers to control the potato cyst nematodes *Globodera rostochiensis* and *G. pallida.* Both of these species are widespread across England, with *G. pallida* now the predominant species (Minnis *et al.*, 2002). *Globodera pallida* can be harder to control than *G. rostochiensis* because of its longer hatch period (Whitehead *et al.*, 1984; Haydock and Evans, 1998). As such, it is important that soil-applied, granular nematicides remain at an effective concentration within the soil long enough to suppress the invasion of potato plant roots by *G. pallida* juveniles. Controlling PCN is costly, an estimated eight million pounds were spent on granular nematicides in 1999 (Evans and Haydock, 2000). Also, the percentage of potato growing land treated with nematicides has changed little since then, an estimated 25% was treated in 1998 and 27% treated in 2002 (Garthwaite *et al.*, 2002). Failure of a granular nematicide as a result of enhanced degradation would not only mean wasted expenditure on the chemical itself but also economic losses as a result of crop failure. It is therefore necessary to be able to predict the occurrence of enhanced degradation within soils. This ability would also prevent unnecessary application, benefiting both the farmer and the environment.

A number of ideas for a diagnostic assay of nematicide persistence have been investigated. Chapter Two of this thesis investigated the possibility of using specific bacterial species, involved in oxamyl degradation, as indicators of enhanced degradation. Bacterial species involved in the degradation of other methylcarbamate nematicides-insecticides have also been investigated (Parekh *et al.*, 1995; Desaint *et al.*, 2000; Karpouzas *et al.*, 2000a).

However, the high degree of diversity amongst isolated methylcarbamate degraders and the uncertainty over whether isolated degraders are actually involved in degradation within the soil suggest that the use of specific bacterial species is not a suitable method (Parekh *et al.*, 1995; Desaint *et al.*, 2000; Desaint *et al.*, 2003). A potentially more effective method is the use of specific methylcarbamate-degradation genes, such as the *mcd* gene, as indicators of enhanced degradation. The *mcd* gene has been isolated from a number of geographically distinct methylcarbamate-degrading bacteria (Topp *et al.*, 1993; Parekh *et al.*, 1995). However, it is not present in all isolated methylcarbamate-degrading bacteria indicating that other, as yet unidentified, degradation genes are involved (Desaint *et al.*, 2000). Presently, the use of a gene probe to quantify oxamyl-degrading bacteria within a soil is only suitable in soils where the majority of degraders possess the *mcd* gene. The gene-probe method will hopefully be a future option when further methylcarbamate-degradation genes have been identified.

Soil OX 11 failed to demonstrate enhanced rates of oxamyl degradation in the incubation study detailed in Chapter Two and also failed to rapidly degrade oxamyl in enrichment culture (Chapter 3). All other soils tested demonstrated enhanced degradation in soil incubation, which was again observed when these soils were tested in enrichment culture. As such, it was suspected that the enrichment culture method might reliably reflect oxamyl degradation patterns recorded in soil incubation and hence enhanced degradation potential in the field. The enrichment culture method would not offer a rapid diagnosis of enhanced degradation potential within a soil, but it is simpler and less laborious than the soil incubation method frequently used to investigate pesticide degradation. This chapter therefore investigates the suitability of the enrichment culture method as a diagnostic assay of nematicide persistence in soils.

4.1.1 Objectives

- To investigate the suitability of using the first enrichment culture cycle as a diagnostic assay for predicting nematicide persistence

- To determine whether enhanced degradation recorded in soil incubation also occurs with the same soil in enrichment culture.

- To determine whether pH-indicator dyes can be used to indicate rapid oxamyl degradation in liquid enrichment culture, thereby simplifying the enrichment culture method by removing the need to monitor the oxamyl concentration.

- To investigate whether oxamyl-degrading bacteria can also degrade the indicator dyes bromcresol purple and bromthymol blue in liquid enrichment culture.

4.2 MATERIALS AND METHODS

4.2.1 Preliminary Experiment: Soil OX 11 Media Test

Further investigations were carried out to confirm that the lack of rapid oxamyl degradation in enrichment culture by soil OX 11 (Chapter 3) was not a result of the complex liquid enrichment media used. To test this, the degradation of oxamyl by soil OX 11 was tested in a number of different media types:

1. Mineral salts medium containing oxamyl at 15 mg l^{-1} (MSM+OX) with an additional nitrogen and carbon source (MSM+OX+C&N)
2. MSM+OX plus an additional carbon source (MSM+OX+C)
3. MSM+OX with no additional nitrogen or carbon source.

4.2.1.1 Media Preparation

Mineral Salts Medium contained the following in g l^{-1}: KH_2PO_4, 2.27; $Na_2HPO_4.12H_2O$, 5.97; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $MnSO_4.4H_2O$, 0.02; $FeSO_4$, 0.005; NaCl, 1.0. A nitrogen source was added to the media in the form of NH_4Cl at 1.0 g l^{-1}. Media containing an additional nitrogen source did not contain NaCl. Media containing an additional carbon source contained filter-sterilised glucose at 1.0 g l^{-1} (Roberts *et al.*, 1991). The procedure for preparing the different media types is described in Section 3.2.1.1. To eliminate methanol from the media, oxamyl was added as 60 μl of a 5 g l^{-1} 50:50 methanol and water stock solution to empty, sterile, 100-ml screw-top bottles. The bottles were left to stand, with the lids removed, for at least 30 min in a microbiological safety cabinet to allow the methanol to evaporate. Twenty millilitres of liquid media was then added to the bottle giving a final oxamyl concentration of 15 mg l^{-1}. All media was mixed by hand shaking.

4.2.1.2 Soil OX11 Enrichment Culture

Soil OX 11 previously received 3 successive oxamyl applications in an incubation study (Chapter 2) and had since been stored at –20°C (approximately 1.5 years). A small amount of soil from OX 11 was removed from the freezer and defrosted over-night at 4°C. Each liquid enrichment medium was inoculated with 0.5 g of soil, with three replicates. Three MSM+OX broths were left uninoculated to act as controls. All cultures were incubated at 25°C in a shaking incubator at 120 rpm.

Cultures were sampled immediately after inoculation and then periodically in order to measure the remaining oxamyl concentration. Sub-samples (0.5 ml) were mixed with an equal amount of methanol to stop microbial degradation before being centrifuged (13000 *g*, 1 min) to remove particles. Sub-samples were stored at -20°C until analysis by HPLC (Section 3.2.1.3).

4.2.2 pH-Indicator Dye Test

4.2.2.1 Preparation of Media

Mineral salts medium supplemented with a nitrogen source (MSMN) was used in the indicator dye test. Oxamyl was added as a solution in 50:50 methanol and water to give a final concentration of 15 mg l^{-1}. The media preparation procedure is described in Section 3.2.1.1 and the methanol elimination method in Section 3.2.6.1.

Bromthymol blue and bromcresol purple were used to indicate pH change. They were added directly to 20 ml of MSMN+OX as a 0.04 % solution in 0.02 M sodium hydroxide that had been sterilised by filtering through a 0.2 μm syringe filter, giving a final dye concentration of 1.5 % (Collins and Lyne, 1970). These two dyes were chosen because their published pH range was within the range that was expected to be observed in the

cultures. Bromthymol blue ranged from blue at pH 7.6 to yellow at pH 6.0, and bromcresol purple ranged from blue at pH 6.8 to yellow at pH 5.8 (Collins and Lyne, 1970). Soil OX 4 (0.5 g) was used to inoculate the bottles because this soil had demonstrated rapid oxamyl degradation in previous enrichment cultures (Chapter 3).

4.2.2.2 Indicator-Dye Test

The suitability of a pH-indicator dye was determined using the following media types:

1. MSMN + OX and bromthymol blue
2. MSMN + OX and bromcresol purple
3. MSMN (no oxamyl) containing bromthymol blue
4. MSMN (no oxamyl) containing bromcresol purple
5. Control: MSMN + oxamyl and bromthymol blue

All media types were tested in triplicate. Media types 3 and 4, that did not contain oxamyl, were set up to determine whether or not the bacteria could utilise the dyes as a carbon source. All cultures were incubated at 25°C in a shaking incubator. Sub-samples were taken immediately after inoculation with soil and thereafter at approximate 3-day intervals. Oxamyl concentration, culture pH and absorbance readings of the indicator dyes were measured at each sampling point.

4.2.2.3 Measuring Oxamyl Concentration

Sub-samples (0.5 ml) were taken from the cultures immediately after inoculation with the soil sample and at intervals thereafter. An equal amount of methanol was added to the sub-sample to stop microbial degradation of oxamyl. The sample was then centrifuged to remove particles before analysis by HPLC. The HPLC method is detailed in Section 3.2.2.1

4.2.2.4 pH

Cultures were sampled (1.0 ml) immediately after inoculation with soil and at intervals thereafter, in addition to the 0.5 ml taken for HPLC analysis. The pH of the 1.0 ml sub-sample was measured using a Russell RL150 pH meter.

4.2.2.5 Dye Concentration

The 1.0 ml sub-sample used for measuring culture pH was re-used to measure the absorbance of the indicator dyes. The sub-sample was centrifuged (16,000 *g*, 1 min) to remove debris before analysis using a Beckman DU-600 spectrophotometer. The spectrophotometer was set to scan between the 400 to 700 nm wavelength. Bromthymol blue absorbance peaked at approximately 615 nm and bromcresol purple absorbance peaked at 588 nm.

4.2.3 Incubation Study

4.2.3.1 Soil Sampling

Soil samples were collected from various locations around England. The same sampling technique used in Section 2.2.1.1 was again employed to control cross-contamination. A contact in Jersey supplied soil from ten fields. These fields were also sampled following the same protocol; however they were not chilled during transit to Harper Adams University College. From each field, 5 kg previously treated and 5 kg previously untreated samples were taken. The previously untreated sample was taken from an area of the same field that had not previously received nematicide applications, usually the headland, verge or hedgerow. All samples were stored at 4°C prior to partial air-drying and sieving through a < 5.6 mm mesh. Soils were refrigerated for no more than 2 weeks. To prevent cross-contamination between soils, sieves that had been sterilised by autoclaving at 121°C for 15 min were used. After processing samples were stored at –20°C.

4.2.3.2 Soil Particle-Size Analysis

Particle-size analysis, organic matter content and pH of all soil samples was determined following the methods previously described in Section 2.2.2.

4.2.3.3 Water-Holding Capacity

All soil samples were adjusted to 30% of their water-holding capacity (WHC) to prevent soil-moisture content from affecting the rate of degradation during incubation. To determine the WHC of each sample, 20 g of air-dried and sieved (< 2 mm) soil was weighed into a Whatman No. 1 filter paper folded to fit inside a funnel. To allow for free drainage, each funnel was placed in the top of a conical flask. The soil samples were saturated with water and then left to drain for 48 h at room temperature. To prevent water loss by evaporation, the conical flasks were stood in a box containing a shallow layer of water and sealed with a lid. After draining, samples (including wet filter paper) were weighed before being placed in a hot-air oven overnight to obtain dry weight. An average weight of ten wet filter papers was calculated which could then be subtracted from the wet soil weight. Thirty percent of the WHC value was then calculated for each soil. This method closely followed that used by Singh *et al.* (2003b) and Walker and Austin (2004).

4.2.3.4 Incubation Study

Frozen, previously treated and previously untreated samples were weighed out in 1 kg amounts (dry weight) into large plastic bags before being left at room temperature to defrost over night. To allow thorough mixing, the soil was spread out inside the plastic bag and oxamyl was applied as 5.4 ml of a 500 mg l^{-1} solution in sterile distilled water (SDW) to give a concentration of 2.7 mg kg^{-1}, approximately equivalent to field rate (5.5 kg ha^{-1}). Soil moisture content was then adjusted to 30% of WHC by the addition of SDW (Table 12). The soil was thoroughly mixed using disposable spoons (one per sample to prevent cross-contamination) before splitting the sample into three replicate 300 g amounts and

incubating in 500-ml, screw-cap, polyethylene containers. All samples were incubated at 15°C and moisture content was maintained by the addition of SDW when required based on sample weight.

To measure the oxamyl concentration, sub-samples (20 g) were taken immediately after oxamyl application and at 5-day intervals from then on. Sub-samples were taken using disposable spoons to prevent cross-contamination. The majority of sub-samples were extracted (Section 4.2.3.5) immediately; however, when oxamyl extraction could not be performed on the same day as sampling, sub-samples were stored at -20°C. Frozen sub-samples were defrosted overnight at 4°C to limit the activity of oxamyl-degrading bacteria prior to extraction.

4.2.3.5 Soil Extraction and Oxamyl Analysis by HPLC

Sub-samples (20 g) were shaken with 20 ml of HPLC-grade methanol for 3 hours on a reciprocating shaker at 300 rpm. One millilitre of supernatant was then centrifuged (16,000 *g* for 1 min) to remove particles before being stored at -20 °C. The analytical efficiency of the extraction procedure is detailed in Section 2.2.5.

Prior to HPLC analysis, all oxamyl extracts were diluted 50:50 with distilled water. Oxamyl analysis was carried out on a Hewlett Packard Series 1100 HPLC using a Phenomenex, 250 x 4.6 mm Sphereclone 5 μm ODS column held at 30°C. The mobile phase was 50:50 HPLC grade water and methanol run at a flow rate of 1.2 ml min^{-1}. A 20-μl injection was monitored at 220 nm with a retention time of 3.5 min. Oxamyl analysis closely followed the method used by Ambrose *et al.* (2000)

Table 12: Soil Physical Properties

Origin	Soil[a]	Particle Size Analysis % sand	% silt	% clay	Soil Type	% OM	pH	moisture content (%)[b]
Glentworth,	1T	74	8	18	Sandy loam	3.5	7.4	19.6
Lincolnshire	1U	64	11	25	Sandy clay	5.5	7.5	20.6
Owmby by Spittal,	2T	69	10	21	Sandy clay	6.80	7.5	17.3
Lincolnshire	2U	68	11	21	Sandy clay	5.0	7.4	20.1
Owmby by Spittal,	3T	62	14	24	Sandy clay	5.3	7.2	18.4
Lincolnshire	3U	64	10	26	Sandy clay	5.9	7.6	20.0
Cromer,	4T	41	46	13	Sandy silt	2.8	6.7	13.1
Norfolk	4U	37	48	15	Sandy silt	3.2	6.3	14.2
Cromer,	5T	45	40	15	Sandy silt	3.0	6.5	14.7
Norfolk	5U	38	48	14	Sandy silt	3.7	6.5	15.5
Penzance,	6T	61	28	11	Sandy loam	4.0	7.1	13.6
Cornwall	6U	65	24	11	Sandy loam	5.8	7.0	16.5
Penzance,	7T	46	46	8	Sandy silt	7.0	6.7	15.1
Cornwall	7U	55	29	16	Sandy loam	8.0	6.9	17.2
Lynn,	8T	82	5	13	Sandy loam	3.0	6.6	14.3
Shropshire	8U	82	9	9	Loamy sand	4.2	5.8	14.9
Chetwynd Heath,	9T	80	9	11	Sandy loam	3.6	6.0	15.8
Shropshire	9U	82	6	12	Sandy loam	3.4	5.6	14.6
Trinity,	10T	35	48	17	Sandy silt	3.6	5.0	17.1
Jersey	10U	31	53	16	Sandy silt	4.2	5.4	17.8
Trinity,	11T	42	41	17	Sandy silt	3.8	5.5	16.5
Jersey	11U	35	49	16	Sandy silt	6.7	4.9	17.2
St. John,	12T	42	45	13	Sandy silt	3.8	4.8	15.4
Jersey	12U	53	35	12	Sandy loam	4.8	5.6	16.5
St. Peter,	13T	63	27	10	Sandy loam	3.0	5.5	13.9
Jersey	13U	58	27	15	Sandy loam	2.8	6.1	14.8
Trinity,	14T	31	44	25	Clay loam	3.8	4.9	17.0
Jersey	14U	31	36	33	Clay loam	4.5	5.3	17.8
Becquet	15T	40	48	12	Sandy silt	3.6	4.8	16.7
Vincent, Jersey	15U	35	53	12	Sandy silt	4.2	5.2	17.6
St. Peter,	16T	79	16	5	Loamy sand	2.7	5.0	13.5
Jersey	16U	62	33	5	Sandy loam	2.9	5.3	14.3
Rozel,	17T	31	61	8	Sandy silt	3.5	5.5	16.6
Jersey	17U	30	56	14	Sandy silt	4.9	6.0	18.0
St. Peter,	18T	71	23	6	Sandy loam	2.7	5.1	14.5
Jersey	18U	64	28	8	Sandy loam	3.5	6.1	15.0
Rozel,	19T	36	53	11	Sandy silt	2.9	5.3	17.1
Jersey	19U	27	64	9	Sandy silt	4.9	5.4	19.7

a- T= *previously treated*, U= *previously untreated*

b- Moisture content at 30% of measured WHC.

4.2.4 Enrichment Culture Study

4.2.4.1 Media Preparation

Mineral Salts Medium (MSM) contained the following in g l^{-1}: KH_2PO_4, 2.27; $Na_2HPO_4.12H_2O$, 5.97; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $MnSO_4.4H_2O$, 0.02; $FeSO_4$, 0.005. The media contained an additional nitrogen source in the form of NH_4Cl, 1.0 g l^{-1} (MSMN). The media preparation procedure is described in Section 3.2.1.1. To eliminate methanol from the media, oxamyl was added as 60 μl of a 5 g l^{-1} 50:50 methanol and water stock solution to empty, sterile, 100-ml screw-top bottles. The bottles were left to stand, with the lids removed, for at least 30 min in a microbiological safety cabinet to allow the methanol to evaporate. Twenty millilitres of MSMN was then added to the bottle giving a final oxamyl concentration of 15 mg l^{-1} (MSMN+OX). The MSMN+OX was then mixed by hand shaking.

4.2.4.2 Enrichment Culture Study

The enrichment culture study commenced one day after the start of the incubation study; therefore there was very little difference between the two experiments in terms of prior soil storage. A small amount of soil from each soil sample was defrosted over night at 4°C. These samples had not received the oxamyl treatment applied at the beginning of the incubation study. Soil (0.5 g) was added to 20 ml MSMN+OX (15 mg l^{-1}) with three replicates for each previously treated and previously untreated sample. Three control replicates consisting of uninoculated MSMN+OX were included to monitor the rate of abiotic degradation. All cultures were incubated at 25°C in a shaking incubator for 30 days.

Sub-samples (0.5 g) were taken immediately after inoculation and at 5-day intervals from then on to monitor the oxamyl concentration. An equal amount of methanol was added to the sub-sample to stop microbial degradation before storage at -20°C. Prior to HPLC

analysis, samples were centrifuged (16,000 *g*, 1 min) to remove particles. The HPLC method for analysing oxamyl concentration is described in Section 3.2.2.1.

4.2.5 Analysis of Results

The time to 50% degradation of the initial oxamyl concentration (DT50) was used to summarise the degradation data for the incubation and enrichment culture experiments. This value was calculated from curves fitted to the raw data for each replicate. The rationale behind the curve fitting followed the principal of parsimony, by which models with the fewest terms and maximum explanatory power were selected first. As such, if a linear regression did not give a good fit, *i.e.*, an r^2 value of 0.95 or more, an exponential or Gompertz curve was fitted. The Gompertz curve was fitted to data that showed a marked delay before rapid degradation occurred (Karpouzas *et al.*, 2001), a degradation pattern seen frequently in the enrichment cultures. All curves were fitted using SigmaPlot version 8.0. Equations for the three curves are shown below:

Linear: $y = y_0 + ax$

Exponential: $y = y_0 + ae^{-bx}$

Gompertz: $y = y_0 + ae^{-e^{-\left(\frac{x-x_0}{b}\right)}}$

To calculate the DT50 value, the Solve function in SigmaPlot version 8.0 was used to return an X axes value from a known Y axes value based on the fitted curve. Significant differences between soils were determined by one-way Analysis of Variance (ANOVA) of the DT50 values from all replicates (n=3) for each soil using Genstat version 5.0. Significance was measured at the 95% level (P<0.05).

4.3.1 Preliminary Experiment: Soil OX 11 Media Test

The soil OX 11 media test showed oxamyl to be degraded most rapidly in media containing an additional carbon source and media containing no additional carbon or nitrogen (Figure 15). In the case of these two media types the time to 50% degradation (12.2 days for both media types) was significantly quicker ($P<0.05$) than the uninoculated control (DT50 of 17.1 days).

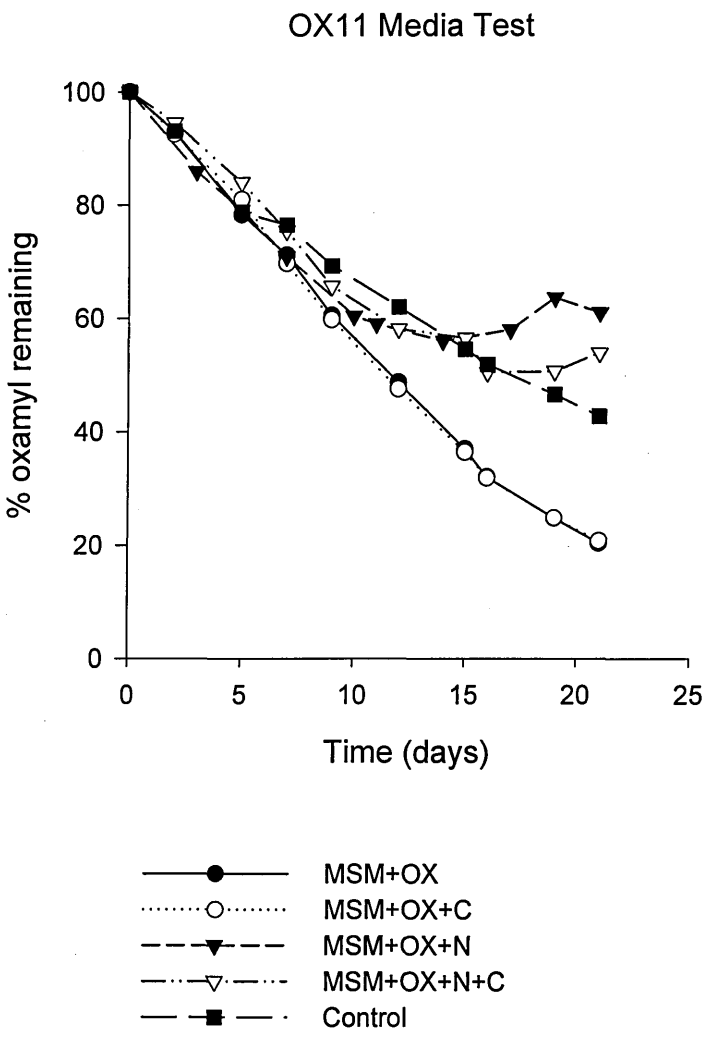


Figure 15: The degradation of oxamyl by soil OX 11 in media containing different nutritional supplements. Data points are the mean of three replicates and are expressed as a percentage of the day 0 value. MSM+OX+N data is from Chapter 3.

Although an increased rate of degradation was observed in the MSM+OX+C and MSM+OX cultures, the linear shape of the degradation curve does not suggest enhanced degradation. As such, MSMN+OX medium was used to test the potential of the enrichment culture method as a diagnostic assay for nematicide persistence (Section 4.2.4). This medium had previously been shown to support the growth of oxamyl-degrading bacteria from different soils (Chapter 3).

4.3.2 pH Indicator Dye Test

In the indicator dye test, the absorbancy of both bromthymol blue and bromcresol purple decreased in all the inoculated broths, whether they contained oxamyl or not, even though there was no corresponding change in pH (Figure 16). A corresponding decreasing trend in the absorbancy readings of bromthymol blue was not observed in the uninoculated controls. The decreasing absorbancy readings recorded for both dyes in cultures that did not contain oxamyl strongly suggests that the bacteria are degrading the dyes, possibly even utilising them as a carbon source. The degradation of the dyes in the inoculated cultures was clearly visible as the dye colour in these broths became very faint compared to the controls. None of the uninoculated control broths contained bromcresol purple; however it is highly likely that bromcresol purple would have behaved similarly to bromthymol blue, in that the absorbancy readings would have remained stable with no decreasing trend.

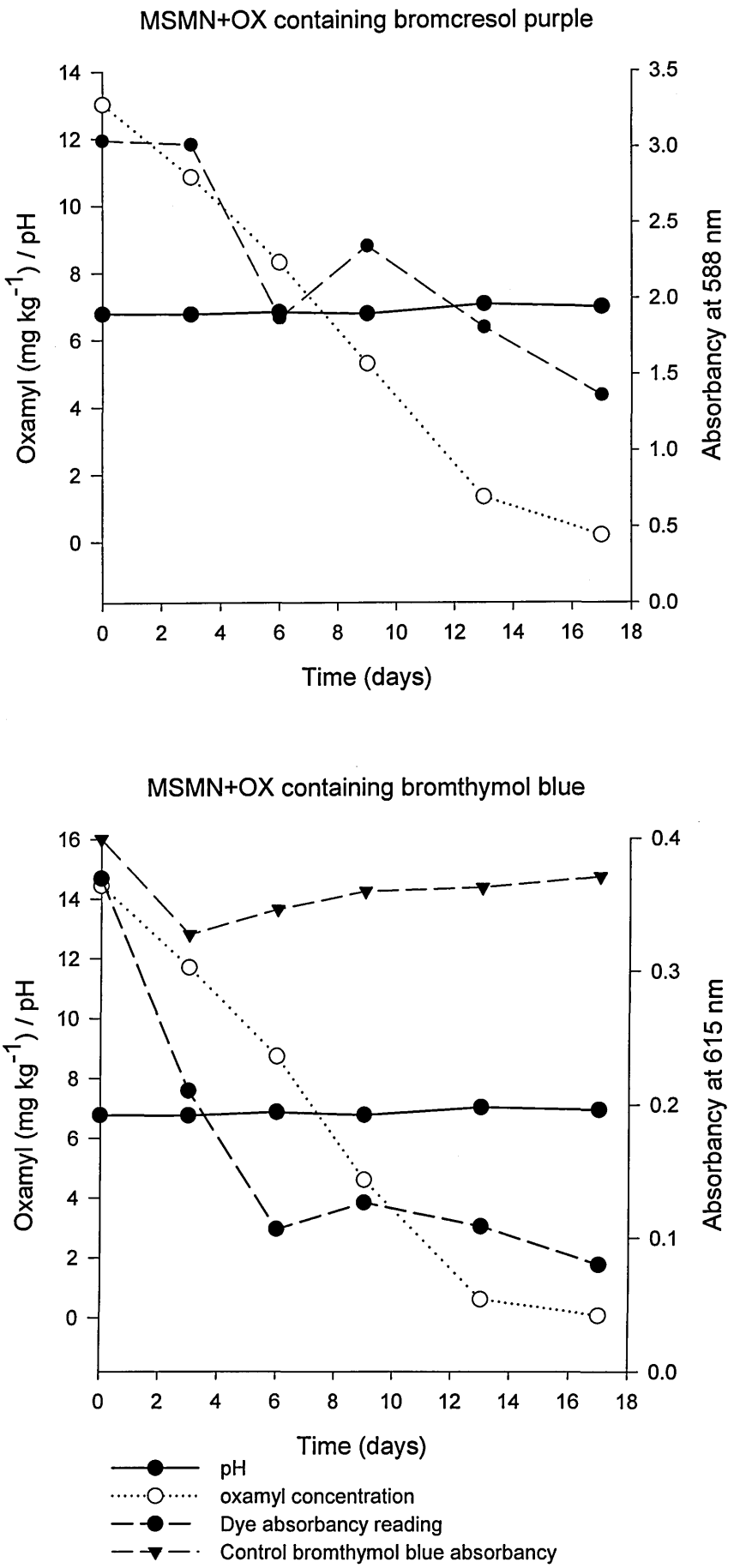


Figure 16: Top: degradation of oxamyl by soil OX4, change in bromcresol purple absorbance measurements over time and culture pH in inoculated MSMN+OX media. Bottom: degradation of oxamyl by soil OX4, change in bromthymol blue absorbance measurements over time and culture pH in inoculated MSMN+OX media.

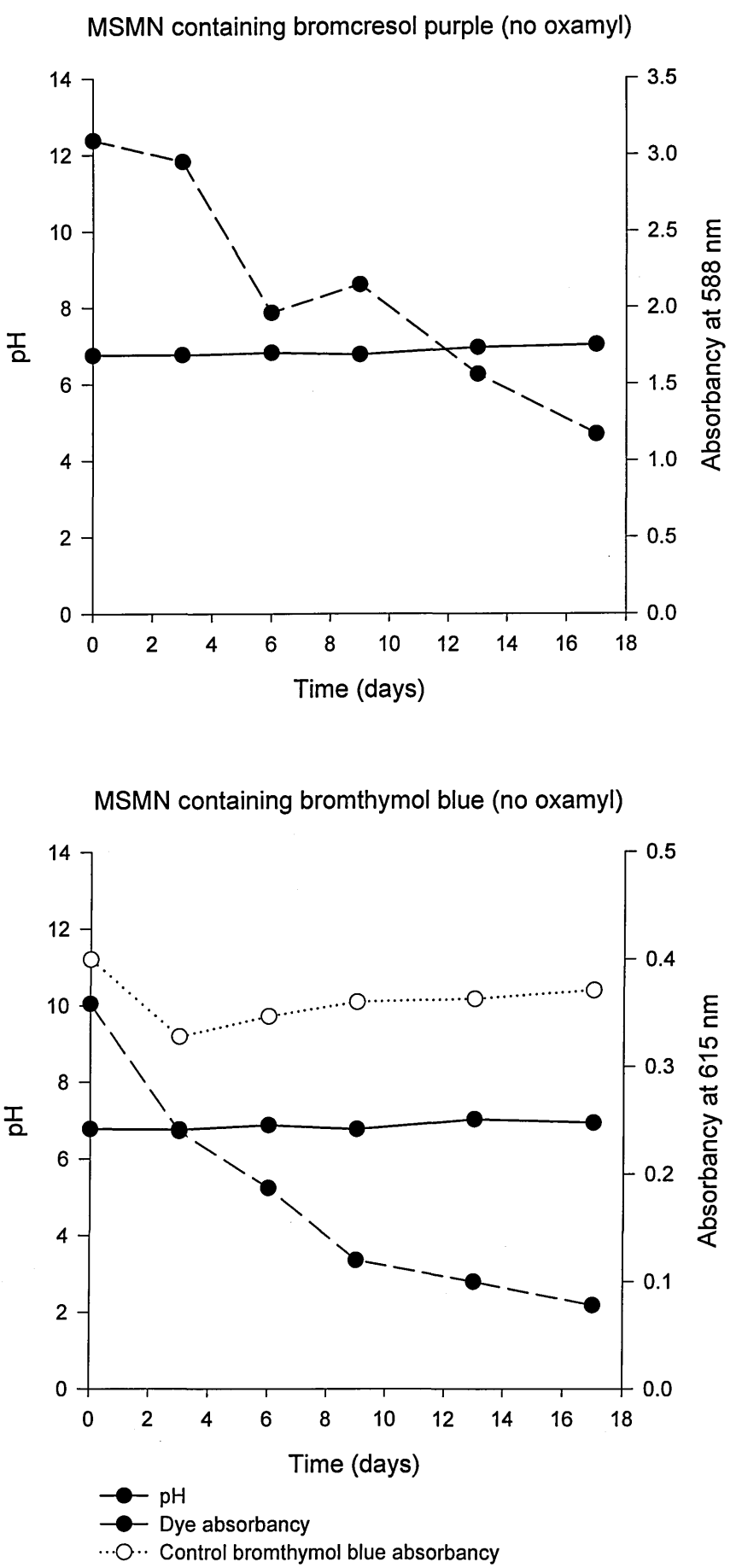


Figure 16 continued: Top: change in bromcresol purple absorbance measurements over time and culture pH in MSMN media inoculated with soil OX4. Bottom: change in bromthymol blue absorbance measurements over time and culture pH in MSMN media inoculated with soil OX4.

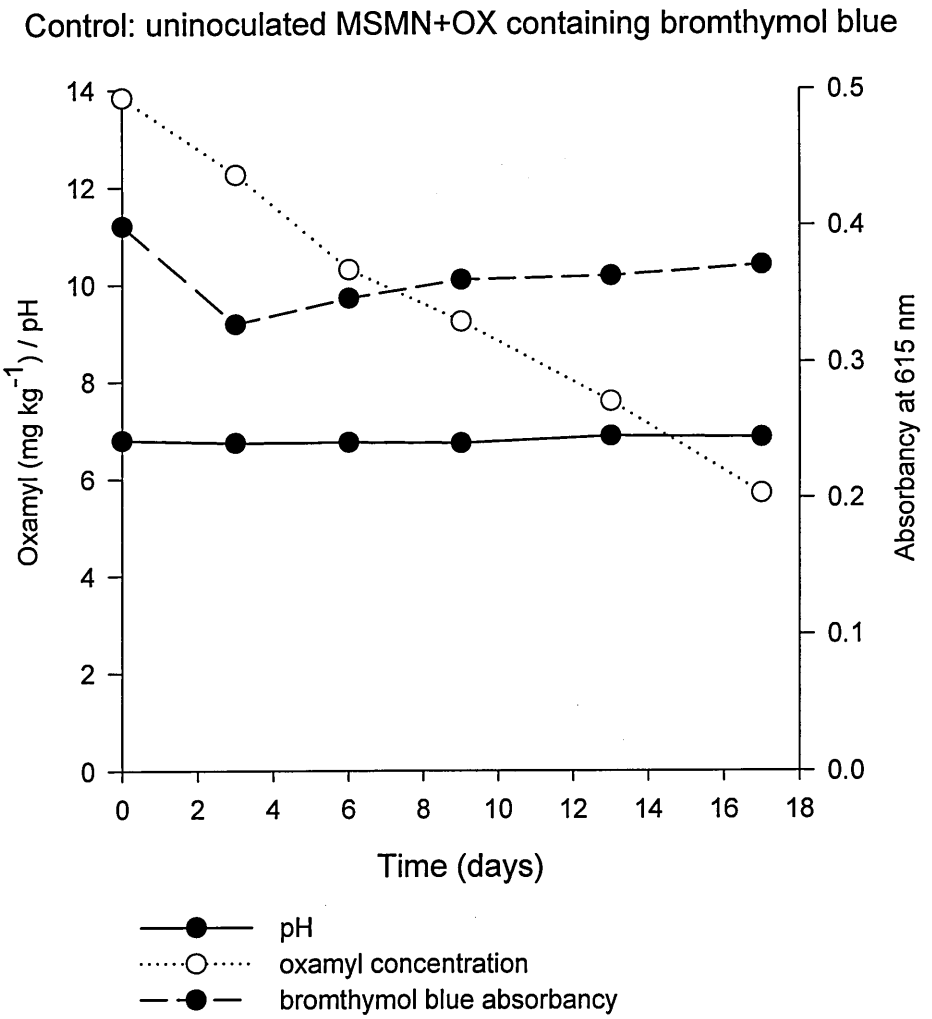


Figure 16 continued: Oxamyl degradation with media pH and bromthymol blue absorbance measurements in uninoculated MSMN+OX.

4.3.3 Enrichment Culture as a Diagnostic Assay

4.3.3.1 Incubation Study

The degradation patterns of oxamyl in previously treated and untreated soils taken from 19 different fields around England and Jersey are displayed in Figure 17. Graphs on the left-hand side show degradation patterns in the soil incubation study and graphs on the right are for the same soil in enrichment culture. From Table 14 it is possible to determine which previously treated soils demonstrated a more rapid rate of oxamyl degradation than their corresponding previously untreated controls based on the time taken to degrade 50% of the applied oxamyl. The DT50 values were calculated by fitting either a linear, exponential or Gompertz curve to the raw oxamyl concentration data for each replicate (complete datasets

for both the incubation and enrichment culture studies are available in Chapter 7, Tables 7.12 and 7.13). The curve that gave the best percentage fit (r^2) was used and the DT50 values calculated from this curve. The mean r^2 values for the fitted curves are displayed in Table 14. It should be noted that as with Chapter 2, the DT50 values were not calculated as first-order half-lives due to the difficulty of applying linear regression to semi-log transformed degradation data for a number of the soils. As a result, the DT50 values are not independent of the concentration, as a half-life would be. Therefore, it can not be assumed that the percentage of pesticide lost per unit time is constant. As such, the DT50 value should not be used to predict the persistence time for concentrations that were not measured in the experiment, *i.e.*, DT50 should not be predicted if the experiment was stopped at the point of 30% degradation (Hamaker *et al.*, 1972). In cases where degradation was slow and the point of 50% degradation was not reached before the end of the experiment, as with some of the Jersey soils, SigmaPlot v. 8.0 calculated the DT50 value by extending the fitted curve beyond the final data point. These values have been included in Table 14; however, they will be subject to a degree of error.

There appears to be a clear divide between the Jersey soils (soils 10-19) and the English soils (soils1-9) regarding the rate of oxamyl degradation, which is clearly evident from the graphs and the DT50 values. Even though all of the Jersey soils had an intensive oxamyl application history of three successive annual applications or more, some with a history of annual application going back to the early 1990s, the rate of degradation amongst the previously treated soils was still low. Only one of the Jersey soils, 16T, degraded 50% of the initial concentration of oxamyl (DT50 of 15.0 days) significantly faster ($P<0.05$) than the previously untreated control (DT50 28.7 days). For soils 14T and 15T, the rate of degradation was so slow that the time taken to degrade only 25% of the initial

Table 13: Nematicide application history of the previously treated soil samples

Soil	*Nematicide application history*	*Rate(kg ai/ha)*
1T	oxamyl 1997, 2003	5.5
2T	oxamyl 1997, 2003	1997- 4.6 2003- 5.1
3T	oxamyl 1997, 2003	1997- 4.6 2003- 5.1
4T	aldicarb 1997, oxamyl 2003	aldicarb- not known oxamyl- 5.5
5T	oxamyl 1998, 2003	5.5 all years
6T	oxamyl 2001, 2002, 2003	5.5 all years
7T	oxamyl 2001, 2002, 2004	5.5 all years
8T	oxamyl 1987, 1991, 1995, 1999, 2003	1987 + 1991- 4.5 1995, 1999 + 2003- 5.5
9T	oxamyl 1988, 1991, 1995, 1999, 2003	5.5 all years
10T	oxamyl 2001, 2002, 2003	3.8 all years
11T	oxamyl 2001, 2002, 2003	Not known
12T	oxamyl every year from 1991 to 2004	3.3
13T	oxamyl every year from 1993 to 2003	Not known
14T	oxamyl 2001, 2002, 2003	3.8
15T	oxamyl every year from 1998 to 2004	3.1
16T	oxamyl every year from 1994 to 2004	3.6
17T	oxamyl every year from 1994 to 2004	4.0
18T	oxamyl every year from 1993 to 2003	3.6
19T	oxamyl every year from 1999 to 2004	4.0

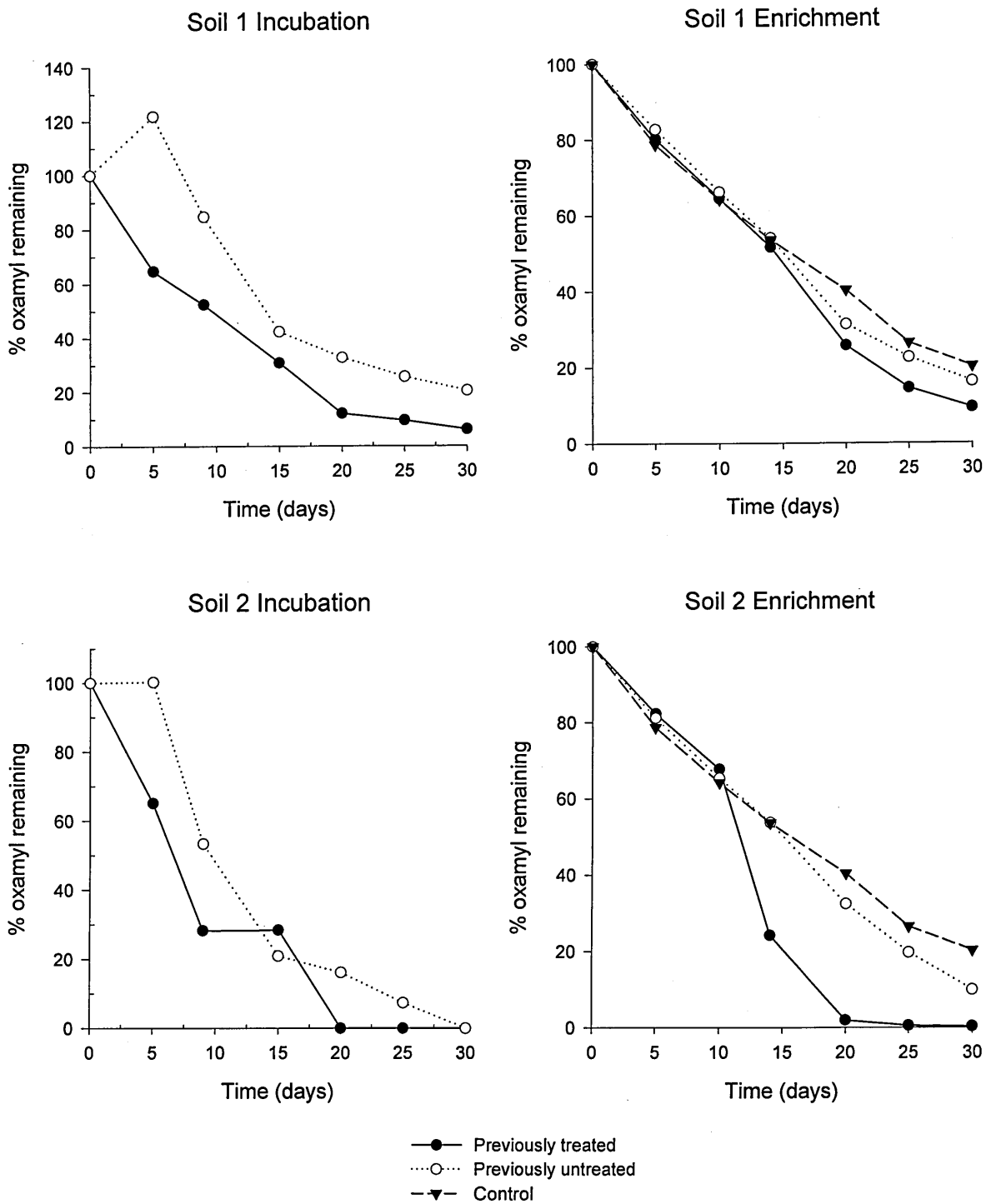


Figure 17: Left hand graphs: degradation of oxamyl in soil 1 and 2 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 1 and 2 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

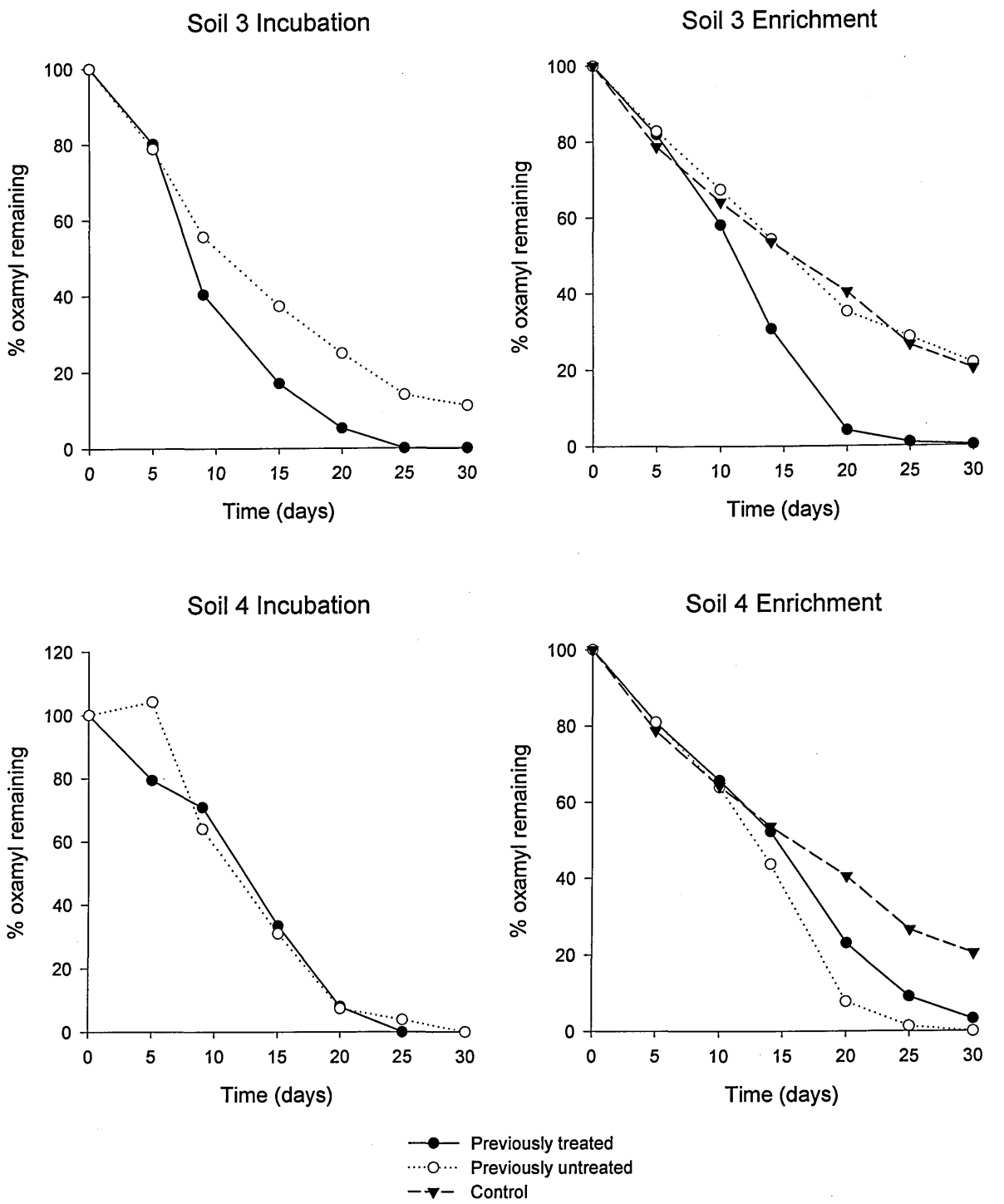


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 3 and 4 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 3 and 4 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

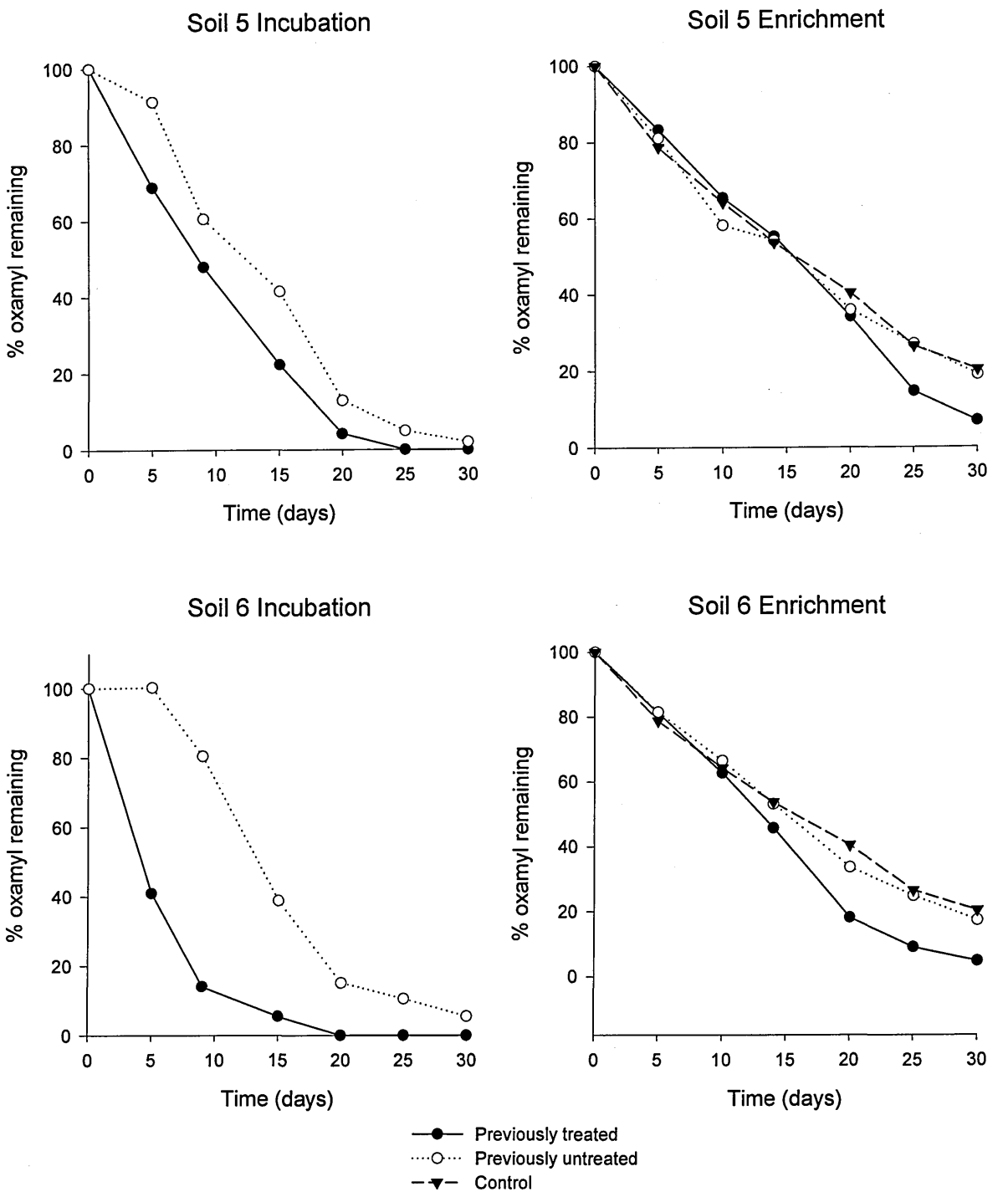


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 5 and 6 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 5 and 6 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

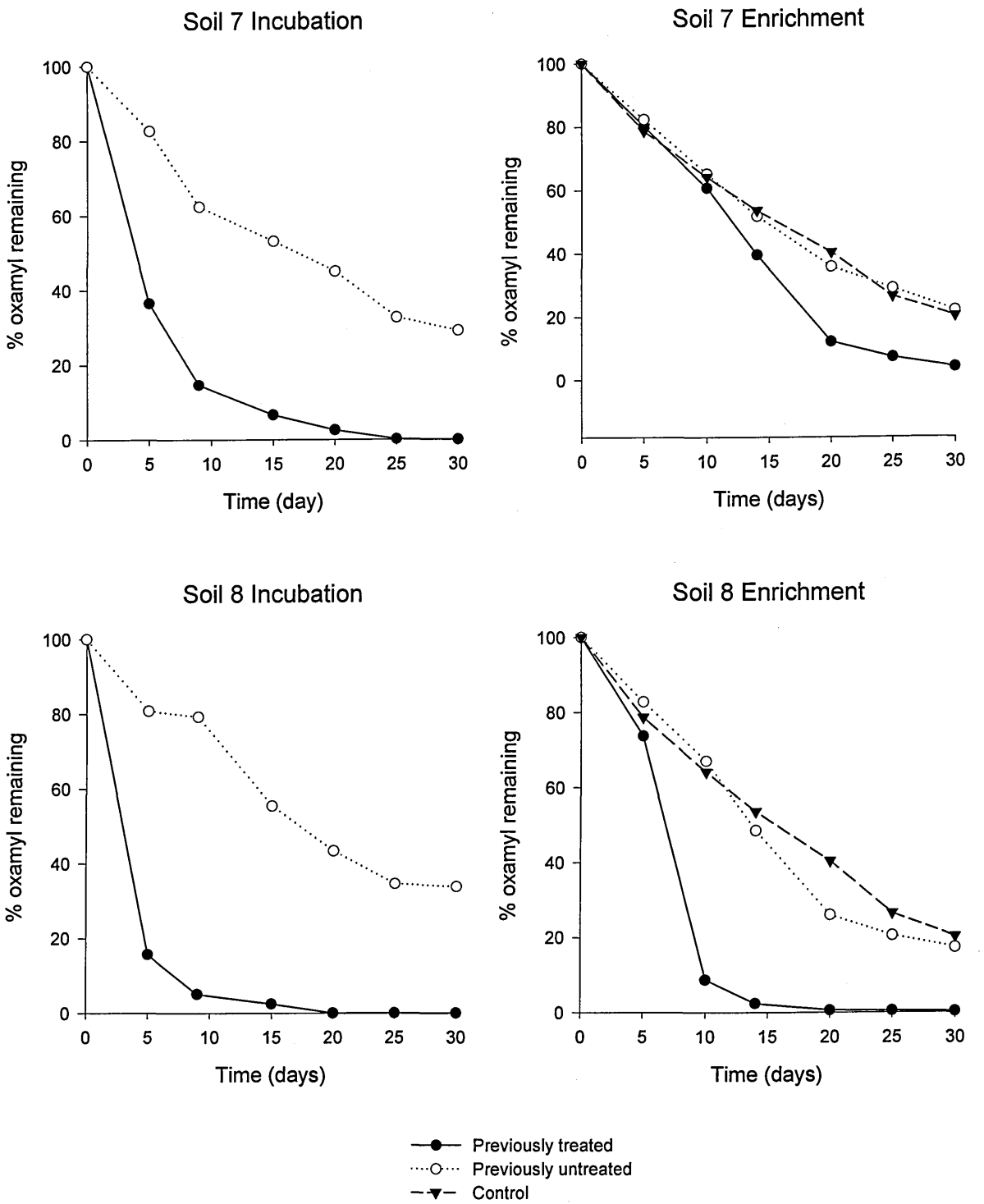


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 7 and 8 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 7 and 8 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

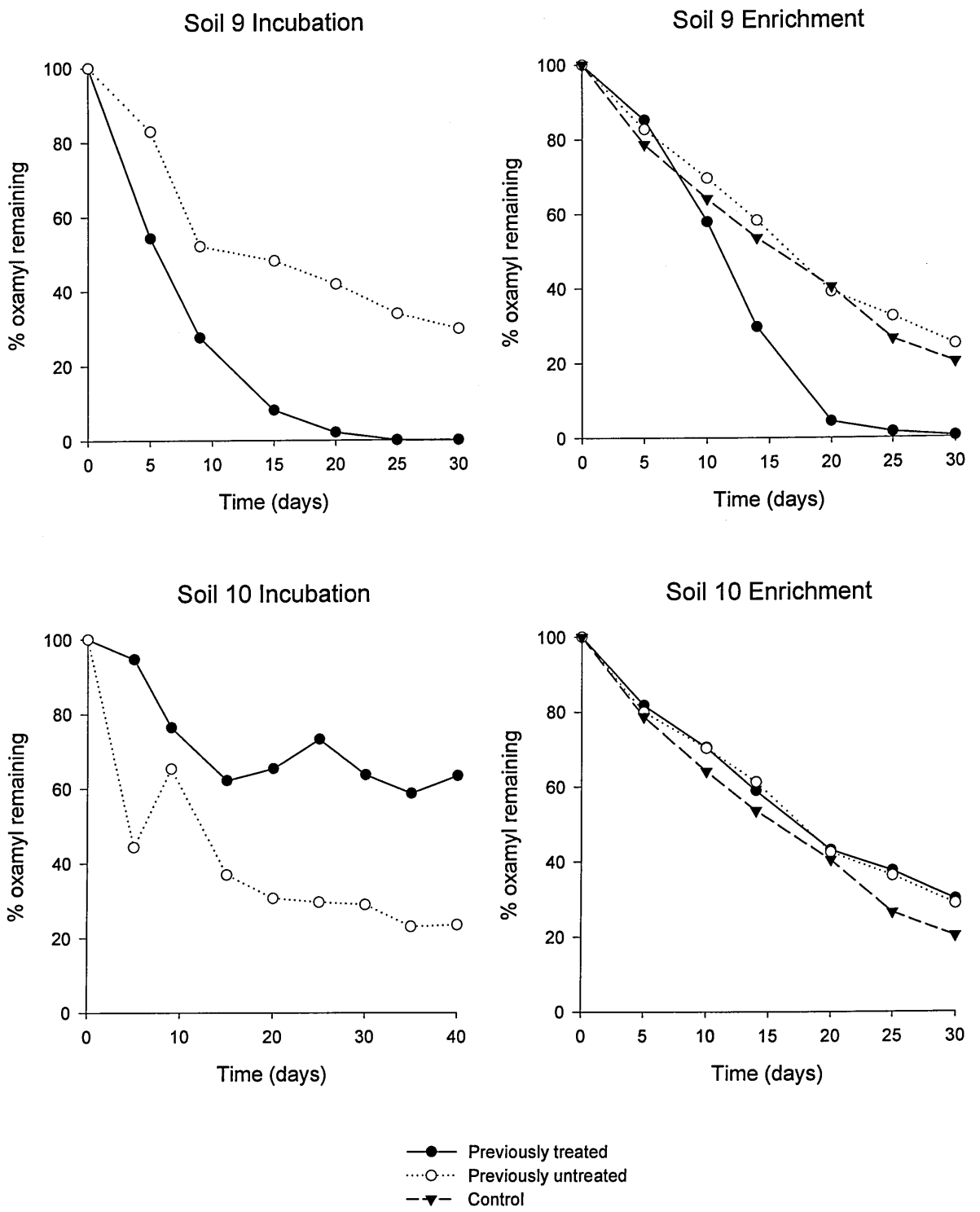


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 9 and 10 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 9 and 10 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

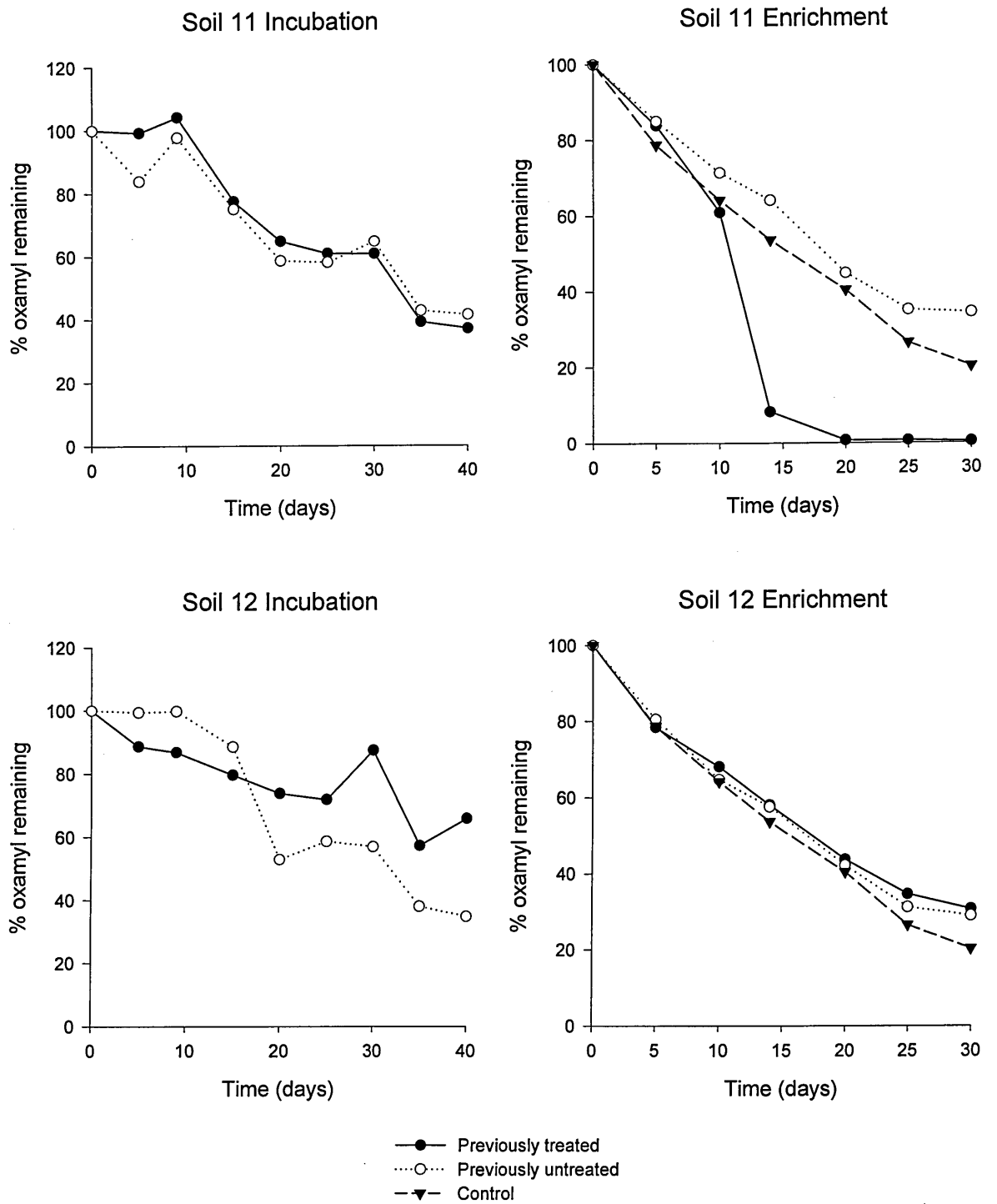


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 11 and 12 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 11 and 12 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

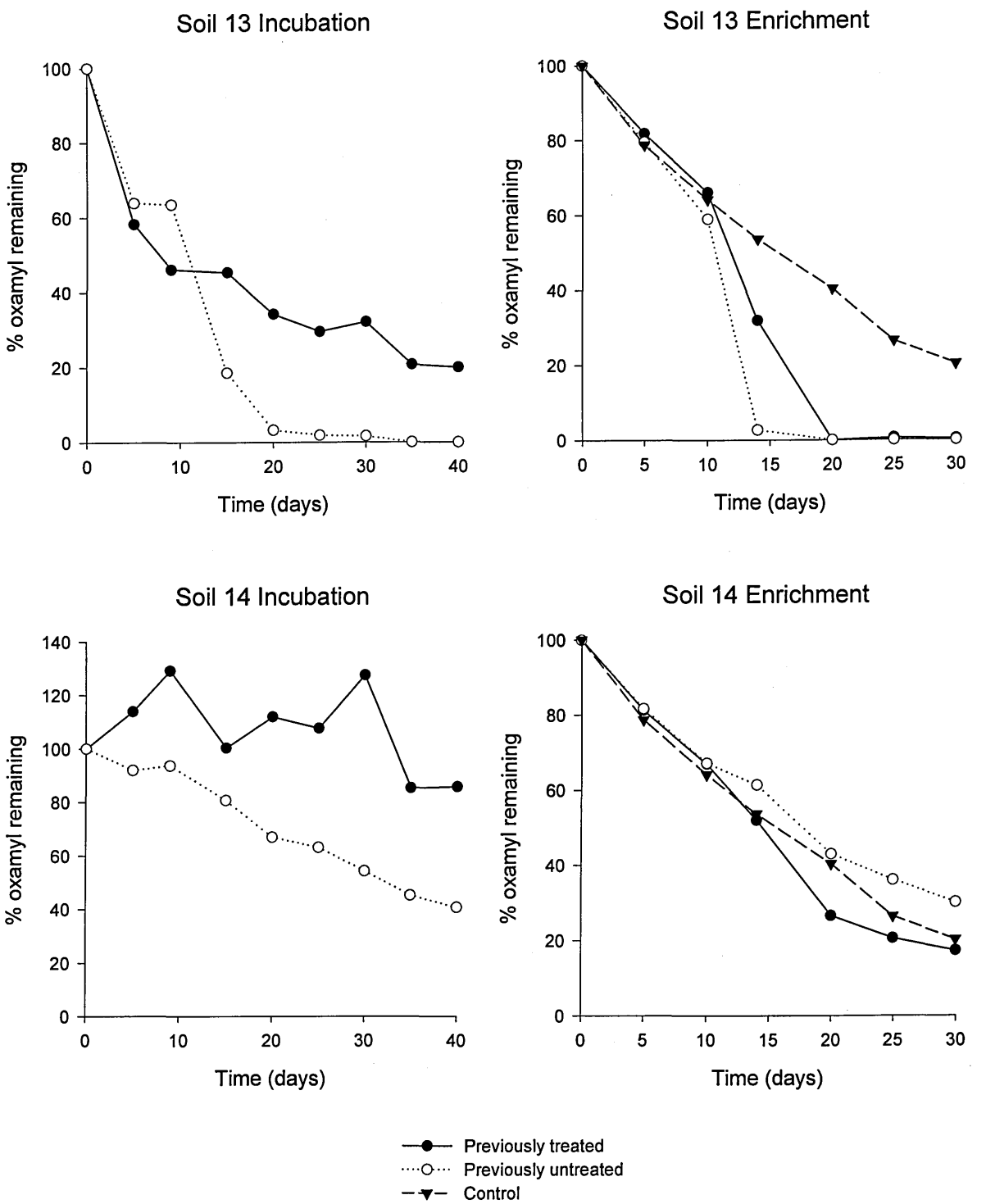


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 13 and 14 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 13 and 14 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

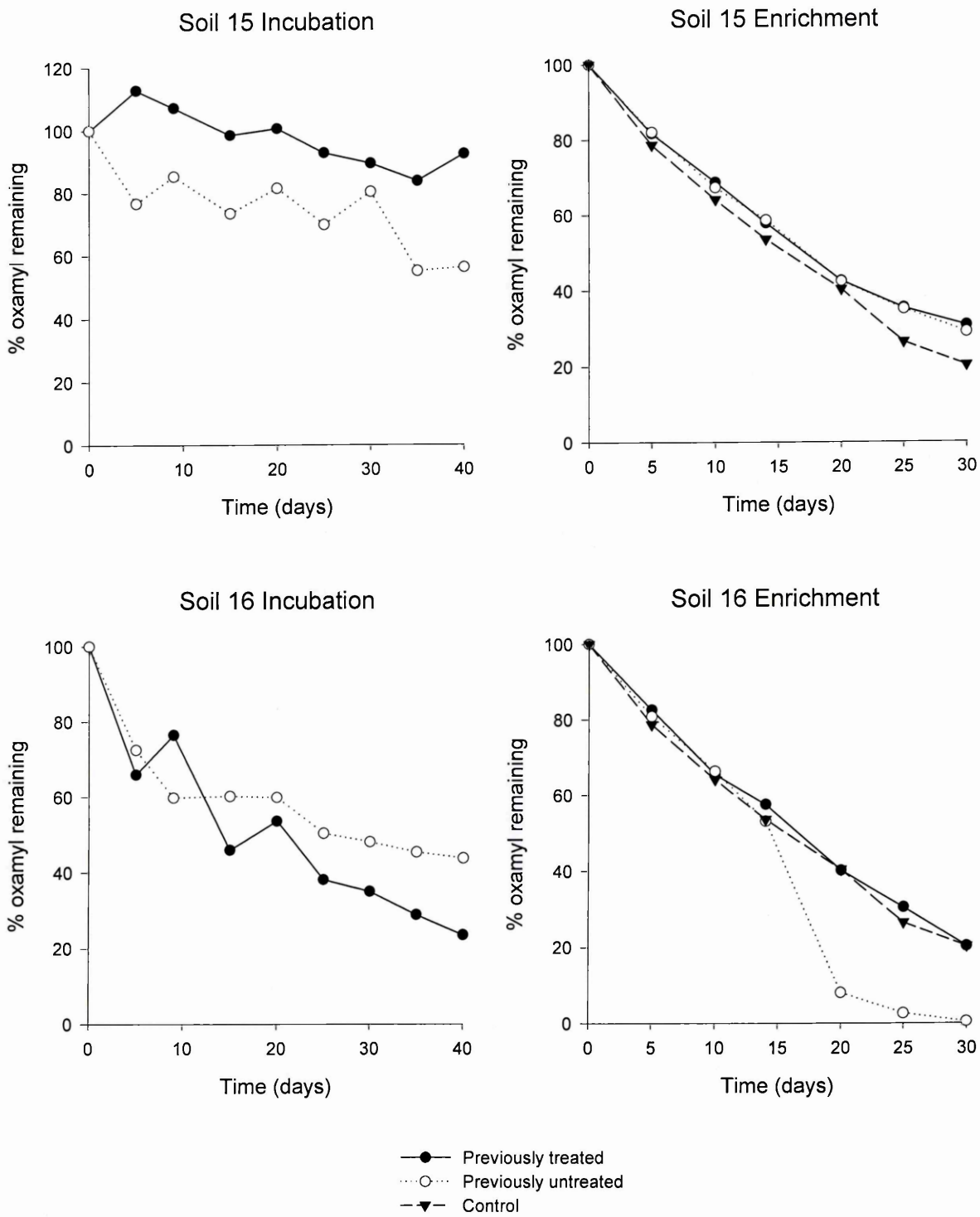


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 15 and 16 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 15 and 16 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

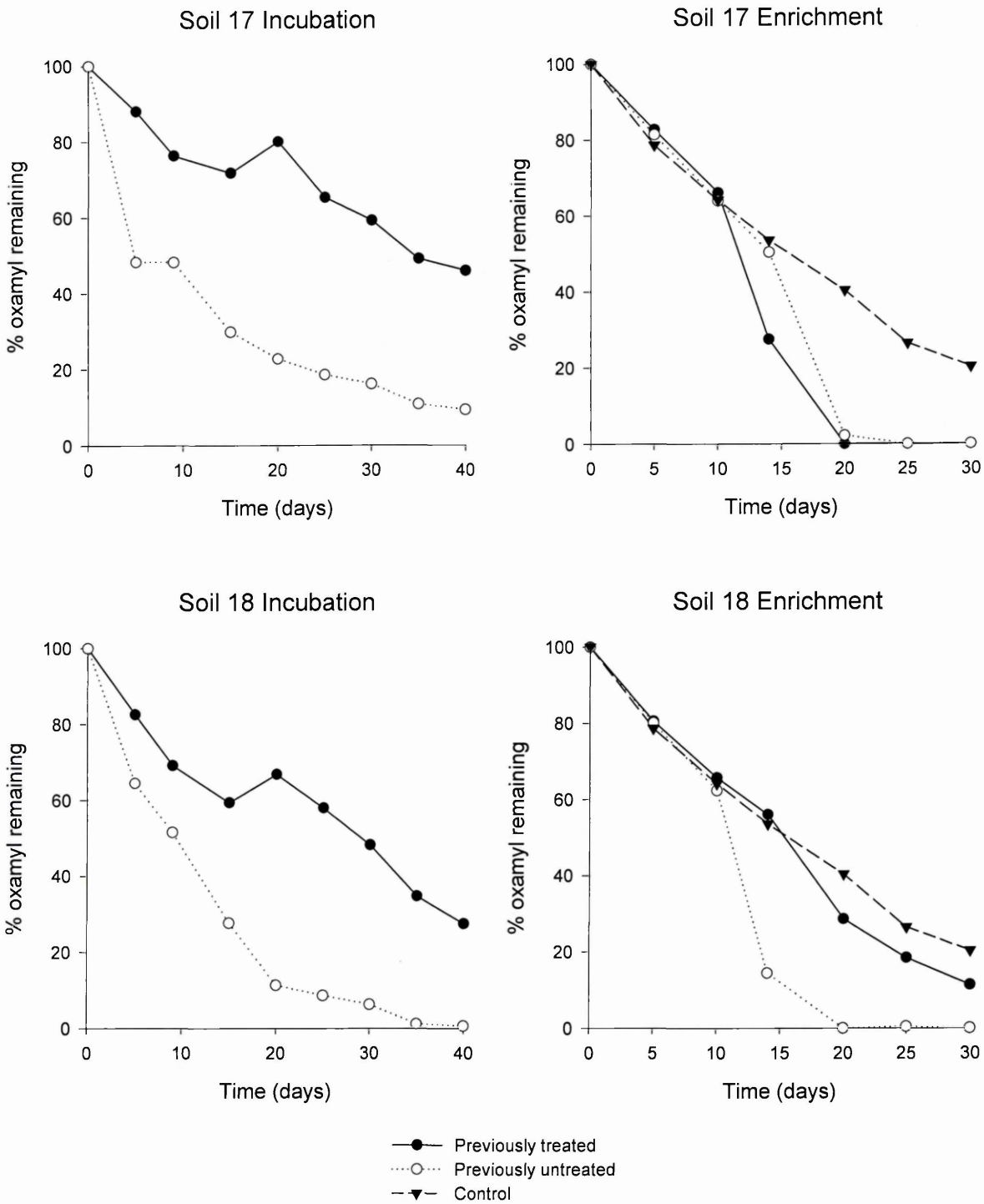


Figure 17 continued: Left hand graphs: degradation of oxamyl in soil 17 and 18 previously treated and previously untreated samples in the soil incubation study. Right hand graphs: degradation of oxamyl in soil 17 and 18 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

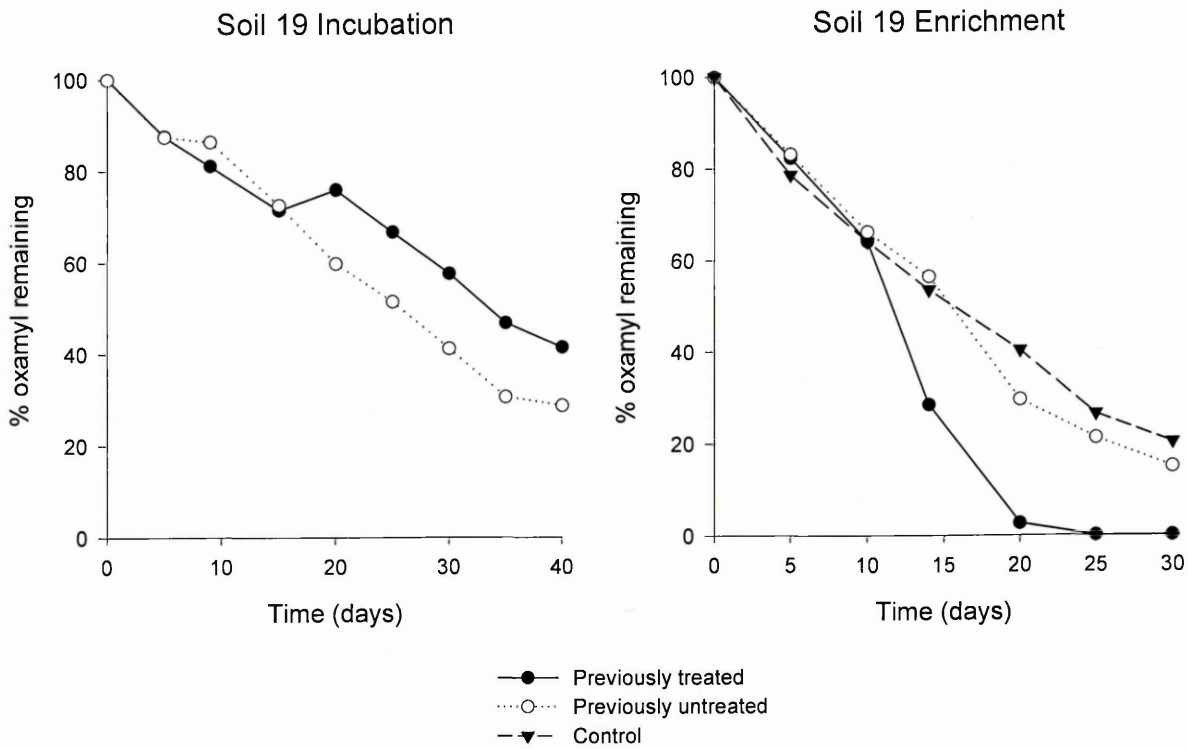


Figure 17 continued: Left hand graph: degradation of oxamyl in soil 19 previously treated and previously untreated samples in the soil incubation study. Right hand graph: degradation of oxamyl in soil 19 previously treated and previously untreated samples in enrichment culture. Data points are mean values (n=3) presented as a percentage of the initial day zero value.

concentration (DT25) had to be measured as the DT50 could not be. All of the previously treated Jersey soils had a pH of 5.5 or less (Table 12). The rate of oxamyl degradation, as determined by DT50 values, was faster in the previously untreated soil for those previously untreated Jersey soils with a higher pH than their corresponding previously treated soil (except soil 16). Soils with the lowest pH (14T, 15T and 12T), also had the largest DT50 values (DT25 in the case of 14T and 15T), 98.0, 64.5 and 56.4 days respectively. Similarly, the Jersey soils with the highest pH, 18U, 17U and 13U, had the lowest DT50 values, 8.2, 8.5 and 9.2 days respectively. The only anomaly was soil 13T, which had a low pH of 5.5 but a comparatively rapid rate of degradation with a DT50 of 9.5 days, although not significantly ($P<0.05$) faster than the corresponding previously untreated soil.

Table 14: Time taken to degrade the applied oxamyl to 50% of the initial concentration (DT50) in the soil incubation study with the corresponding r^2 values for the fitted curves.

Previously treated sample	*pH*	*DT50* [a] *(days)*	R^2 [a]	*Previously untreated sample*	*pH*	*DT50* [a] *(days)*	R^2 [a]
1T	7.4	8.6 (±0.3)	0.99 ($\pm5.04^{-3}$)	1U	7.5	16.5 (±2.2)	0.7 (±0.14)
2T	7.5	6.6 (±0.3)	0.95 (±0.02)	2U	7.4	10.7 (±1.1)	0.91 (±0.03)
3T	7.2	8.5 (±0.9)	0.99 ($\pm3.9^{-3}$)	3U	7.6	10.5 (±2.6)	0.88 (±0.04)
4T	6.7	11.6 (±1.6)	0.99 ($\pm1.8^{-3}$)	4U	6.3	12.2 (±0.4)	0.97 (±0.01)
5T	6.5	8.5 (±1.0)	0.99 ($\pm2.3^{-3}$)	5U	6.5	12.4 (±0.5)	0.99 ($\pm5.8^{-3}$)
6T	7.1	3.6 (±0.2)	0.99 ($\pm5.7^{-3}$)	6U	7.0	13.9 (±0.5)	0.98 ($\pm3.1^{-3}$)
7T	6.7	3.4 (±0.1)	0.99 ($\pm3.3^{-4}$)	7U	6.9	15.9 (±0.8)	0.99 ($\pm1.7^{-3}$)
8T	6.6	1.9 (±0.1)	0.99 ($\pm1.7^{-3}$)	8U	5.8	17.2 (±3.1)	0.95 (±0.01)
9T	6.0	5.1 (±0.2)	0.99 ($\pm2.2^{-3}$)	9U	5.6	13.0 (±2.6)	0.90 (±0.09)
10T	5.0	47.9 (±9.6)	0.50 (±0.08)	10U	5.4	14.6 (±2.6)	0.62 (±0.01)
11T	5.5	31.8 (±1.2)	0.82 (±0.08)	11U	4.9	33.0 (±1.1)	0.84 (±0.06)
12T	4.8	56.4 (±1.4)	0.52 (±0.03)	12U	5.6	30.5 (±0.7)	0.85 (±0.04)
13T	5.5	9.5 (±2.4)	0.95 (±0.02)	13U	6.1	9.2 (±1.7)	0.97 (±0.03)
14T	4.9	98.0* (±28.1)	0.16 (±0.08)	14U	5.3	33.1 (±1.0)	0.88 (±0.07)
15T	4.8	64.5* (±7.7)	0.58 (±0.09)	15U	5.2	19.7* (±5.9)	0.57 (±0.06)
16T	5.0	15.0 (±2.7)	0.91 (±0.02)	16U	5.3	28.7 (±1.7)	0.72 (±0.06)
17T	5.5	36.4 (±1.5)	0.87 (±0.05)	17U	6.0	8.5 (±1.5)	0.94 (±0.03)
18T	5.1	26.7 (±0.8)	0.86 (±0.05)	18U	6.1	8.2 (±0.5)	0.99 ($\pm3.8^{-3}$)
19T	5.3	34.6 (±0.6)	0.89 (±0.03)	19U	5.4	26.3 (±0.6)	0.94 (±0.02)

a The data are mean values (n=3) with standard error
** Values are for DT25 as DT50 could not be calculated*

Table 15: Time taken to degrade the applied oxamyl to 50% of the initial concentration (DT50) in the enrichment culture study with the corresponding r^2 values for the fitted curves.

Previously treated sample	***DT50*** [a] ***(days)***	***R^2*** [a]	***Previously untreated sample***	***DT50*** [a] ***(days)***	***R^2*** [a]
1T	14.8 (±1.1)	0.98 ($\pm5.00^{-4}$)	1U	16.2 (±1.1)	0.97 ($\pm1.45^{-3}$)
2T	10.6 (±0.1)	0.99 (±0.00)	2U	15.4 (±0.2)	0.99 ($\pm2.03^{-3}$)
3T	11.1 (±0.1)	0.99 (±0.00)	3U	13.8 (±1.6)	0.95 (±0.05)
4T	14.2 (±0.1)	0.98 ($\pm8.82^{-4}$)	4U	12.5 (±0.4)	0.99 ($\pm1.53^{-3}$)
5T	15.3 (±0.3)	0.98 ($\pm3.84^{-3}$)	5U	16.2 (±0.3)	0.95 (±0.03)
6T	13.7 (±0.0)	0.97 ($\pm4.41^{-3}$)	6U	16.2 (±0.3)	0.98 ($\pm5.77^{-3}$)
7T	12.8 (±1.0)	0.97 ($\pm8.82^{-3}$)	7U	16.3 (±0.3)	0.97 ($\pm1.22^{-8}$)
8T	6.9 (±0.1)	0.99 ($\pm8.60^{-9}$)	8U	15.8 (±2.1)	0.98 ($\pm5.77^{-3}$)
9T	11.2 (±0.2)	0.99 ($\pm8.60^{-9}$)	9U	18.1 (±0.4)	0.98 ($\pm3.33^{-4}$)
10T	19.3 (±0.3)	0.97 ($\pm5.69^{-3}$)	10U	19.0 (±0.2)	0.98 ($\pm2.66^{-3}$)
11T	10.7 (±0.4)	0.99 ($\pm1.68^{-3}$)	11U	20.1 (±0.7)	0.97 ($\pm1.18^{-3}$)
12T	18.8 (±0.4)	0.97 ($\pm4.44^{-3}$)	12U	18.1 (±0.4)	0.96 ($\pm4.44^{-3}$)
13T	11.8 (±0.3)	0.99 ($\pm3.28^{-4}$)	13U	10.4 (±0.3)	0.99 ($\pm1.51^{-3}$)
14T	14.8 (±1.0)	0.99 ($\pm1.2^{e-3}$)	14U	19.1 (±0.5)	0.97 ($\pm4.02^{-3}$)
15T	19.0 (±0.2)	0.97 ($\pm1.16^{-3}$)	15U	18.7 (±0.2)	0.97 ($\pm3.26^{-3}$)
16T	17.4 (±0.2)	0.98 ($\pm8.61^{-3}$)	16U	13.6 (±0.3)	0.99 ($\pm3.85^{-3}$)
17T	11.6 (±0.1)	0.99 ($\pm1.67^{-4}$)	17U	13.0 (±0.2)	0.99 ($\pm2.00^{-3}$)
18T	15.7 (±1.0)	0.99 ($\pm2.95^{-3}$)	18U	10.8 (±0.0)	0.99 ($\pm3.38^{-4}$)
19T	11.8 (±0.8)	0.99 ($\pm1.03^{-3}$)	19U	16.4 (±1.2)	0.98 ($\pm3.49^{-3}$)
Control	16.2 (±0.3)	0.97 ($\pm7.90^{-3}$)			

a The data are mean values (n=3) with standard error

Table 16: Significant differences (P<0.05) between the DT50 values of previously treated and untreated soils in incubation and enrichment. Also, significant differences between previously treated soils and the uninoculated control in enrichment.

Soil	*T significantly faster than U in incubation?* [a]	*T significantly faster than U in enrichment?* [a]	*T significantly faster than control in enrichment?* [a]
1	Yes		
2	Yes	Yes	Yes
3		Yes	Yes
4			Yes
5	Yes		
6	Yes	Yes	Yes
7	Yes	Yes	Yes
8	Yes	Yes	Yes
9	Yes	Yes	Yes
10			
11		Yes	Yes
12			
13			Yes
14		Yes	
15			
16	Yes		
17		Yes	Yes
18			
19		Yes	Yes

[a] T = previously treated, U = previously untreated
Where difference is not significant, the column is left blank.

In contrast, soils 1-9 taken from fields around England demonstrated much more rapid oxamyl degradation with DT50 values as low as 1.9 days (soil 8T). The majority of the previously treated English soils demonstrated significantly faster degradation than their corresponding previously untreated soil, the exceptions being 3T and 4T. The degradation graph for 3T does, however, suggest that due to the sigmoid shape of the degradation curve oxamyl is being degraded faster. By day 25, soil 3T had completely degraded the initially applied oxamyl, whereas more than 10% still remained by day 30 in soil 3U. In contrast to the Jersey soils, the pH values of the English soils are much higher, ranging from 5.6 in soil 9U to 7.6 in 3U. Also dissimilar to the Jersey soils, there appears to be less connection between DT50 and pH as the English soils with the higher pH values do not necessarily

have the lowest DT50 values. For example, soils 8T, 7T, and 9T had DT50s of 1.9, 3.4 and 5.1 days respectively but pH values of only 6.6, 6.7 and 6.0. Soils 8T and 9T did however have an intensive history of oxamyl application, with applications made every 4 years since 1987 and 1988 respectively (Table 13). Similarly, soil 7T received 3 successive annual applications of oxamyl from 2001 to 2003.

4.3.3.2 Enrichment Culture Study

The degradation patterns of oxamyl in the enrichment cultures are displayed in the right hand graphs in Figure 17 and the mean DT50 values for both the previously treated and previously untreated soils are shown in Table 15. Significant differences between the previously treated soils and the previously untreated soils, and also the previously treated soils and the uninoculated controls, are displayed in Table 16. The mineral salts media used in the enrichment culture is designed to maintain a constant pH at around 6.8 and as such the differences in degradation rate between the Jersey (10-19) and English soils (1-9) are not as marked as in the incubation study. Even so, six of the nine (67%) previously treated English soils degraded 50% of the initial oxamyl concentration significantly faster than the corresponding previously untreated soils, whereas only four of the ten (40%) previously treated Jersey soils were significantly faster than their corresponding previously untreated. All of the significantly faster, previously treated soils from both England and Jersey, except soil 14T, were also significantly faster than the uninoculated controls. For soils 4, 13, 16 and 18, however, the rate of degradation as determined by DT50 values was actually significantly faster in the previously untreated sample. This reflects the incubation study results for soils 4, 13 and 18, where there was either no significant difference between previously treated and previously untreated samples or the previously untreated sample degraded oxamyl significantly faster. Although 4T and 13T were not significantly faster than the corresponding previously untreated samples in enrichment, both were

significantly faster than the control, suggesting that oxamyl-degrading bacteria were present in both the previously treated and untreated samples.

4.3.3.3 Incubation and Enrichment Results Compared

By comparing the results from the incubation study and enrichment study, it is possible to determine whether the enrichment culture reflects the degradation patterns observed in incubation, and thus is a good method for predicting the occurrence of enhanced degradation. Of the 19 previously treated soils, five demonstrated significantly faster oxamyl degradation than the previously untreated sample in both the incubation and the enrichment studies. These soils, soils 2, 6, 7, 8 and 9, were all taken from locations in England. Soil 2 came from a field in Lincolnshire, soils 6 and 7 came from two different fields in Cornwall and soils 8 and 9 were taken from two different fields in Shropshire. All of the previously treated samples for these fields had a pH of 6.0 or above (Table 12). For these soils, the enrichment culture method does appear to reflect the occurrence of enhanced degradation as seen in the incubation study. Similarly, soils 10T, 12T, 15T and 18T failed to demonstrate enhanced degradation in both the incubation study and the enrichment study, providing further support for the reliability of the diagnostic assay.

A number of previously treated soils (3T, 11T, 14T, 17T and 19T) degraded oxamyl significantly faster than the corresponding previously untreated sample in enrichment culture, even though this was not the case in the incubation study. Similarly, soils 1T, 5T and 16T degraded oxamyl significantly faster than the corresponding previously untreated soil in incubation but not in enrichment. Also, these three soils were not significantly faster than the control in enrichment. In the case of significantly faster degradation in enrichment but not in incubation, all of these soils, except 3T, were from Jersey and had low pH values of 5.5 or less. From Figure 17 it can be seen that there is no indication of enhanced degradation in incubation for these four Jersey soils (11T, 14T, 17T and 19T), the

previously untreated sample being seen to degrade oxamyl faster than the previously treated in a number of these soils.

4.4 DISCUSSION

It was initially thought that indicator dyes could be used to signal rapid oxamyl degradation within enrichment culture by changing colour as the pH of the culture dropped due to bacterial growth. This would simplify and reduce the cost of the enrichment culture method by removing the need to continually monitor the oxamyl concentration using an expensive HPLC assay. However, results from the indicator dye test demonstrated that the soil bacteria present within the soil inoculum (soil OX 4) were not only capable of degrading oxamyl but also the indicator-dyes bromthymol blue and bromcresol purple. The colour of the dyes became progressively fainter, which would of course, prevent any determination of colour change as a result of a change in the pH of the culture. It is quite obvious that the indicator-dye test would not make a suitable diagnostic assay for nematicide persistence in soils and thus the method was not developed further. As a result of the unsuitability of the dye method, the enrichment culture itself was tested as a possible diagnostic assay.

Although not a rapid method, complete degradation of 15 mg kg^{-1} of oxamyl usually takes at least 20-30 days in rapidly degrading cultures,and the enrichment culture method is less laborious than the soil incubation method. There is no lengthy oxamyl extraction procedure, less soil preparation is required beforehand and it is quicker and easier to set up and sample. The enrichment culture method is already being used instead of the soil incubation method by some researchers to investigate mineralisation rates of pesticides (Sorensen and Aamand, 2003). Also, results from a number of the soils tested showed it to be a reliable method. Five soils (soils 2T, 6T, 7T, 8T and 9T) that demonstrated enhanced

degradation in the incubation study also exhibited enhanced degradation in the enrichment culture, and, conversely, enhanced degradation was absent from both incubation and enrichment in soils 10T, 12T, 15T and 18T. This was not, however, the case for all of the soils tested, particularly the Jersey soils (soils 10-19).

It is generally accepted that pesticide degradation is faster in soils with a history of application with the same or similar chemicals than in previously untreated soils (Suett, 1986; Smelt *et al.*, 1987; Karpouzas *et al.*, 1999a). It was then a surprise that the ten soils taken from fields around Jersey failed to demonstrate rapid oxamyl degradation as all had a history of intensive oxamyl application, in some cases annual application going back for more than 10 years. Only soil 16T out of the 10 Jersey soils demonstrated a rate of oxamyl degradation faster than the previously untreated sample in soil incubation, although this soil failed to rapidly degrade oxamyl in enrichment culture. The previously untreated soils 13U and 18U were seen to rapidly degrade oxamyl instead of the corresponding previously treated samples in both incubation and enrichment. Both of these soils had a pH of 6.1, the highest out of the 20 samples taken from Jersey. The pH of all the previously treated Jersey soils was low, ranging from 4.8 to 5.5. Many studies have demonstrated the strong effect of pH on the rate of degradation of carbamate pesticides, with degradation proceeding at a slower rate at lower soil pH (Harvey and Han, 1978; Smelt *et al.*, 1979; Read, 1987; Jones and Norris, 1998; Walker *et al.*, 2001, Singh *et al.*, 2003b). The low pH of the Jersey soils is most likely the cause of the lack of enhanced degradation. Low pH appears to have a greater influence on the development of enhanced degradation than an intensive oxamyl application history. This is consistent with results from other degradation studies. Smelt *et al.* (1996) failed to record enhanced degradation for the nematicides aldicarb, oxamyl and ethoprophos in low pH plots (5.6) that had received annual applications between 1978 and 1987. Similarly, Karpouzas *et al.* (1999a) reported slow degradation of ethoprophos in a soil that had received annual ethoprophos applications for seven years; and also slow

degradation of both ethoprophos and carbofuran in a soil that had received seven successive, annual applications of both chemicals. The authors stated that the slow degradation was almost certainly a result of the low pH of these soils (4.4-4.5). The influence of pH on enhanced degradation is also evident in the enrichment cultures for soils 11T, 17T and 19T. These soils demonstrated a more rapid rate of oxamyl degradation than the corresponding previously untreated sample in enrichment but not in the soil incubation study. The pH of the enrichment culture media is approximately 6.8. This more favourable pH will have allowed the growth of oxamyl-degrading bacteria that were present in soils 11T, 17T and 19T but could not proliferate and metabolise oxamyl in the soil incubation because of the adverse soil pH. This effect of pH on the adaptation of pesticide-degrading bacteria has been documented previously. Bending *et al.* (2003) investigated the effect of pH on two *Sphingomonas* sp. strains capable of degrading isoproturon. The strains had been isolated from a field displaying in-field spatial variability in the degradation of the herbicide. It was found that the strains had a very narrow pH range (7.0-7.5) within which rapid growth-linked degradation of isoproturon could occur, so resulting in the observed variation in isoproturon degradation across the field. Similarly, Suett *et al.* (1996b) found only small numbers of carbofuran-degrading organisms in a previously treated soil of pH 5.4 but much higher numbers in a previously treated soil of pH 7.5. Singh *et al.* (2003a) inoculated enhanced chlorpyrifos-degrading soil into UK soils that had previously shown slower rates of chlorpyrifos degradation. Although this enhanced the rate of degradation in all the UK soils tested, this was short lived in the low-pH soils (4.7 and 5.7) which could not sustain the introduced population of chlorpyrifos degraders. Also of interest from this study (Singh *et al.*, 2003a) were the effects of artificially increasing the soil pH of these low acidic soils by the addition of lime. Increasing the pH from 4.7 to 7.5 and 5.7 to 8.6 resulted in rates of degradation similar to those observed in the soils originally of high pH, further demonstrating the connection between soil pH and degradation rate. Karpouzas and Walker (2000c) reported the rapid

degradation of ethoprophos by two *Pseudomonas putida* isolates in liquid media with a pH range of 5.5 to 7.6. However, rapid degradation was inhibited below pH 5.0.

Interpretation of degradation data can often be quite complex (Walker *et al.*, 2001). A popular way of analysing degradation curves is to compare the time taken to degrade 50% of the initial concentration of the pesticide (DT50) (Walker *et al.*, 1993; Karpouzas *et al.*, 2001; Walker *et al.*, 2001), as was done with the experiment reported in this chapter. However, use of only one time point does not always give a true reflection of the differences between curves. For example, the mean DT50 values for soil samples 3T and 3U in the incubation study were 8.5 and 10.5 days respectively and there was no significant difference between the two. However, analysis of variance of a later degradation point, such as DT60 or DT70, may have resulted in a significant difference between the two samples, as the degradation curves suggest that the difference increased after the 50% point.

The shape of the degradation curves, the DT50 values and the statistical analysis need to be assessed together for correct interpretation of the data to be achieved. The statistical information can not be relied upon solely because a lack of significant difference does not necessarily mean that enhanced degradation has not occurred. In some soils there was no significant difference between previously treated and untreated samples because enhanced degradation had occurred in both, such as soil 4 in both the incubation study and the enrichment culture, and soils 13 and 17 in the enrichment culture study. In the case of these soils, enhanced degradation can be determined from the shape of the degradation curves. A sigmoid-shaped curve, in which an initially slow period of degradation is followed by a rapid rate of degradation, indicates that bacteria are adapting by way of population growth, gene dissemination or enzyme synthesis before a noticeable effect on the rate of degradation can occur. The bacteria are utilising the chemical as an energy or nutrient

source and thus an increase in the size of the population will result in an increased rate of biodegradation (Kearney and Kellogg, 1985). Similarly, a significant difference between previously treated and untreated samples does not necessarily mean enhanced degradation has occurred. The DT50 values for soils 1T and 5T were significantly different from the corresponding previously untreated samples in the incubation study but not significantly different in enrichment culture, suggesting that adapted bacteria were either not present in these soils or they could not survive in the enrichment media used. The shape of the degradation curves for these two soils in soil incubation indicate that degradation was by co-metabolism rather than mineralisation as there was no phase of rapid degradation (Bending *et al.*, 2003). That is, the bacteria are not utilising the chemical and as such enhanced degradation would not be expected to occur (Felsot and Shelton, 1993).

The previously untreated soils used in both the soil incubation study and the enrichment culture study were taken from an area of the same field as the previously treated sample that had never before been treated with oxamyl, such as the headland, field edge or hedgerow. This is a common procedure for enhanced degradation studies (Suett and Jukes, 1988; Roberts *et al.*, 1991; Walker and Welch, 1992; Walker *et al.*, 1993; Karpouzas *et al.*, 1999a). Although the previously untreated sample will not be identical to the previously treated sample, they should be similar. Out of the 19 pairs of soils tested, only four were not classified as the same soil type (Table 12). The use of sterilised soils as controls does have its advantages in that a clear distinction between abiotic (such as pH) and biotic effects on the rate of degradation can be observed (Smelt *et al.*, 1987). However, these effects can not be observed together in both the previously treated and untreated samples, *i.e.*, the effects of co-metabolism on the rate of degradation in the previously untreated soils can not be observed because soil microorganisms are absent. Sterilised soils may have been of use in some of the more alkaline English soils in which the alkaline conditions could have contributed to oxamyl degradation (Wolfe *et al.*, 1990). However, the

degradation data for the majority of the English soils that displayed enhanced degradation suggests that degradation was chiefly microbial. Either an initial lag period was seen prior to rapid degradation, suggesting the bacteria are adapting in someway prior to enhanced degradation (Kearney and Kellogg, 1985), or the rate of degradation was rapid from the outset, indicating that adapted bacteria were already present within the soil as is commonly seen in repeat-application studies (Torstensson, 1980; Felsot and Shelton, 1993).

There is, of course, always concern that degradation data recorded in the stable and constant laboratory environment is not directly transferable to the variable field environment where soil moisture content and temperature will have an effect on the rate of degradation (Suett and Jukes, 1988; Bromilow, 1996). As such, even if enhanced degradation is observed in both the enrichment culture study and the incubation study, it does not then automatically mean that enhanced degradation will occur in the field. As the Jersey soils have shown, at low pH the enrichment method can give false positives, in that rapid degradation can occur in enrichment that would not occur in soil incubation or in the field because of the unfavourable pH. Soil pH would have to be taken into account for all samples analysed and soils with a low pH assumed that enhanced degradation is less likely to occur even if the enrichment culture data shows otherwise. Further work is required to better establish the point at which pH starts to have a suppressive effect on enhanced degradation. Similarly, false positive results could occur with high organic matter soils when tested in liquid enrichment culture. Due to the higher adsorption in high organic matter soils the bioavailability of the pesticide is reduced and thus enhanced degradation is less likely to occur (Walker, 1989; Weber *et al.*, 1993; Guo *et al.*, 1999). However, the adsorption effect of soil organic matter would not occur in liquid enrichment and thus enhanced degradation may occur that would not occur in the field.

An alternative option could be to alter the buffering capacity of the liquid enrichment media to the pH of each soil tested by changing the amounts of KH_2PO_4 and Na_2HPO_4 in the media. This would then show whether oxamyl-degrading bacteria, that may be present in the soil, could degrade oxamyl at the pH that would be encountered in the field. However, in addition to complicating the method further, this would also prevent the enrichment culture method from indicating the presence of oxamyl-degraders within low pH soils. If a farmer was to increase the pH of the field soil by adding lime, enhanced degradation may occur. Two low-pH soils, that had failed to develop enhanced degradation with repeat fenamiphos application, displayed enhanced degradation when their pH was artificially increased by the addition of lime (Singh *et al.*, 2003b). The enrichment culture buffered at a low pH would not allow the outcome of liming to be predicted because it would not indicate the presence of oxamyl-degrading bacteria. In addition, this modification would fail to take into account spatial variability in degradation rates across a field as a result of fluctuations in pH as have been reported by Bending *et al.* (2003). As such, the enrichment culture method may be better used as an indication of the presence of microbes in the soil that are capable of mineralising oxamyl or capable of adapting their metabolism to utilise oxamyl. Thus, enhanced degradation could occur if soil conditions such as pH were ideal for growth of these organisms. This would give a similar indication as would be achieved with a species-specific or gene-specific probe, although the enrichment method would not quantify the degrading bacteria. The more complicated and laborious most probable number method could be used to measure the number of oxamyl-degrading bacteria already present within a soil sample (Roberts *et al.*, 1998; Ostrofsky *et al.*, 2002).

In light of research demonstrating the in-field spatial variability of enhanced degradation (Walker *et al.*, 2001; Bending *et al.*, 2003), the question is raised regarding how many soil samples should be analysed from one field to allow a sound judgement on a field's

potential to rapidly degrade oxamyl? One sample alone is unlikely to be sufficient. Analysis of a number of samples taken from various points throughout the field would be more representative (Walker and Austin, 2004) but more time consuming. Further investigation is required using an experimental set-up similar to that used by Walker *et al.* (2001) to determine the most representative and efficient method. The enrichment culture method could, however, be simplified further using microtitre plates to allow easier analysis of a large amount of samples (Roberts *et al.*, 1998).

The difficulty of interpreting the degradation data, the poor reliability of the method with low-pH soils and the length of incubation mean that the enrichment culture method is not ideal. It may, however, be a useful assay in the short term until more methylcarbamate degradation genes can be identified and sequenced, enabling the use of degradation-gene specific DNA probes or primers.

4.4.1 Conclusions

- Five of the nine previously treated English agricultural soils with a pH of 6.0 or higher demonstrated enhanced degradation in both the soil incubation study and the enrichment culture study. Similarly, four of the soils that failed to demonstrate enhanced degradation in incubation also failed to rapidly degrade oxamyl in enrichment culture.

- The enrichment culture method is less reliable in low pH soils where oxamyl-degrading bacteria may be present but unable to grow and utilise oxamyl in the unfavourable soil environment. The effect of soil pH would need to be considered alongside enrichment culture data for every soil sample analysed.

- The enrichment culture method may be better used as an indicator of the presence of oxamyl-degrading bacteria within the soil that could, if abiotic conditions were favourable, cause enhanced degradation of oxamyl in the field.

- Due to the difficulty of interpreting degradation data, the unreliability of the enrichment method in low pH soils and the lengthy incubation period, the enrichment culture method is not ideal. It may however, be a useful short-term solution until more suitable methods, such as degradation-gene specific DNA probes, can be developed.

5. GENERAL DISCUSSION

5.1 THE POTENTIAL FOR ENHANCED DEGRADATION OF NEMATICIDES IN UK AGRICULTURAL SOILS

The potential for enhanced degradation of the carbamates oxamyl and aldicarb was confirmed in incubation studies using agricultural soils collected from different fields around the UK (Chapters 2 and 4). The rate of degradation of these two carbamates was seen to increase with subsequent applications in some soils, providing evidence for the involvement of adapted bacteria. One soil, soil OX 10, demonstrated enhanced rates of oxamyl degradation after one application in the laboratory and all three successive applications were completely degraded in less than ten days (Chapter 2). Similarly, in Chapter 4 enhanced rates of oxamyl degradation were observed in previously treated UK agricultural soils after one laboratory application when compared to previously untreated soils. This is consistent with previous research that also reported the enhanced degradation of these nematicides. Smelt *et al.* (1996) recorded enhanced rates of oxamyl and aldicarb degradation in samples taken from plots that had received three previous treatments. Ambrose *et al.* (2000) monitored the degradation of oxamyl in the field and found it to be undetectable 21 days after incorporation at one site, demonstrating that rapid oxamyl degradation is not unique to the laboratory and does occur out in the field. However, potential for enhanced degradation of the organophosphorus nematicide fosthiazate was not observed in any of the ten soils tested (Chapter 2). Enhanced degradation was absent regardless of soil type, soil pH, organic matter content and previous treatment history.

Soil physicochemical characteristics, such as soil pH and organic matter content, have an effect on a soil's potential for enhanced degradation. The rate of degradation of a large number of pesticides has been found to decrease with decreasing pH (Smelt *et al.*, 1996; Suett *et al.*, 1996; Qin *et al.*, 2004). Hydrolysis of phosphate and carbamate ester bonds

does tend to occur more rapidly at high pH (Wolfe *et al.*, 1990). However, pH also has an effect on soil bacteria. Bending *et al.* (2003) investigated the microbial mechanisms behind the spatial variablility of isoproturon degradation observed in a UK field. They found that the isolated *Sphingomonas* spp. strains capable of rapid isoproturon degradation had a narrow optimum pH, ranging from 7.0 to 7.5, within which they could mineralise the herbicide. This probably accounted for the variability in degradation within the field. Similarly, inoculation of chlorpyrifos-degrading bacteria into soils that had previously failed to demonstrate enhanced degradation resulted in increased rates of degradation in only those soils with a more neutral to alkaline pH, degradation remained slow in the lower pH soils (Singh *et al.*, 2003a). There appeared to be less potential for enhanced degradation of oxamyl within the low pH soils sampled from fields in Jersey (Chapter 4). Here, soils that had an intensive history of annual application but a low pH of 5.5 or less demonstrated slow rates of oxamyl degradation; in many cases this was slower than the previously untreated soil. However, when tested in liquid enrichment culture with a stable pH of approximately 6.8, rapid degradation was observed in some of these soils demonstrating that microorganisms capable of oxamyl degradation were present within the soils. The unfavourable pH conditions of the soils could have had an inhibitive effect on these organisms, resulting in the slow rate of degradation observed in the soil incubation study. Less of a pH effect was observed in the incubation study described in Chapter 2, although slower rates of aldicarb and oxamyl degradation were recorded in the lower pH soils. Enhanced degradation of aldicarb also appeared to be affected by organic matter content (Chapter 2). Parent aldicarb and aldicarb sulphoxide were seen to be more persistent in the three soils containing a large amount of organic matter. Increased aldicarb persistence in high organic matter soils has been reported previously (Smelt *et al.*, 1978a,b,c; Guo *et al.*, 1999).

Concern over the enhanced degradation of nematicides in UK agricultural soils does appear to be well founded as the research presented in this thesis has demonstrated the potential for enhanced degradation of the carbamates aldicarb and oxamyl in UK soils. In light of this, what can be done to prevent enhanced degradation occurring? Although cross-enhancement was not tested directly in Chapter 2, a lack of enhanced degradation of the organophosphate fosthiazate was observed, whereas the carbamates oxamyl and aldicarb were seen to be more susceptible to enhanced rates of degradation. As previous research has demonstrated that cross-enhancement between different chemical groups is rare (Smelt *et al.*, 1987; Smelt *et al.*, 1996; Karpouzas *et al.*, 2004), a possible suggestion would be that fosthiazate be used in instances where the efficacy of oxamyl or aldicarb has been reduced as a result of enhanced degradation. Rotation between nematicides from different chemical groups could be a successful preventative measure (Karpouzas and Giannakou, 2002).

Of further interest is the fortuitous coincidence of low pH in the intensely used Jersey soils (Chapter 4). Potatoes are an important crop on the island and are grown annually. All of the Jersey fields sampled had a history of annual oxamyl application, dating back to the early 1990s in some cases. As the enrichment culture data from Chapter 4 has shown, some of these soils did contain microorganisms capable of rapidly mineralising oxamyl; however, the low pH of the soils has most probably protected the nematicide from enhanced degradation in the field, preventing the deleterious effects of PCN invasion. Enhanced degradation would otherwise have been expected to occur in these soils due to the intensive treatment history.

5.2 PREDICTING ENHANCED DEGRADATION

If it is not possible to implement preventative measures to reduce the possibility of enhanced degradation, or preventative measures are not succesful, an assay designed to predict a soils potential for enhanced degradation of applied nematicides may be of use. One possibility is the quantification of specific bacterial species, adapted to degrade certain pesticides, within soil samples. Enhanced oxamyl degradation within soils does appear to be mediated by adapted soil bacteria and, for the first time, bacteria capable of oxamyl degradation have been isolated from soils demonstrating potential for enhanced oxamyl degradation (Chapter 3). The 36 oxamyl-degrading isolates were assigned to only four different species. Twenty-one of these isolates demonstrated similarity to the same species, *Aminobacter aminovorans*, indicating that perhaps quantification of this species within soils could give an indication of the potential for enhanced degradation. However, previous research has demonstrated the high degree of genetic diversity amongst bacterial isolates capable of degrading carbamate pesticides (Parekh *et al.*, 1995; Desaint *et al.*, 2000). Although the lack of diversity reported in Chapter 3 is not unique, Rousseaux *et al.* (2001) also failed to report a high degree of diversity amongst atrazine-degrading isolates, it is unlikely that this lack of diversity is truly representative of what is occurring in the soil. It is suspected that the enrichment culture method selects for fast-growing organisms adapted to the complex media used (Karpouzas *et al.*, 2000a). As such, a diagnostic assay involving specific bacterial species may not be appropriate.

Although the evidence suggests that a species specific diagnostic assay is not suitable, the isolation of oxamyl-degrading bacteria has potentially helped to further knowledge of the bacterial species involved in pesticide degradation, particularly as *Mesorhizobium* sp. isolates capable of pesticide degradation have not previously been isolated from enhanced degrading soils. Also, the isolates could be of potential use in bioremediation studies, where adapted bacteria are used to 'clean-up' contaminated sites. A number of the studies

investigating pesticide-degrading bacteria have done so with the problem of pesticide contamination of soil and water in mind (Chaudhry and Wheeler, 1988; Chapalamadugu and Chaudhry, 1993; Yanze-Kontchou and Gschwind, 1994; Topp *et al.*, 2000;). Approaches to bioremediation with microorganisms focus on two main techniques. Firstly, applying the microorganisms directly to the contaminated site and, secondly, using purified enzyme preparations originating from pesticide-degrading microbes for the purpose of decontamination (Chapalamadugu and Chaudhry, 1992). Inoculation of an atrazine-degrading *Agrobacterium radiobacter* isolate into soil that had not previously displayed enhanced rates of atrazine degradation resulted in two to five times more atrazine mineralisation (Struthers *et al.*, 1998). Singh *et al.* (2004) achieved similar results when they applied a chlorpyrifos-degrading *Enterobacter* strain to a soil with an originally low indigenous population of chlorpyrifos degraders. There have been reports of aldicarb contamination of ground water, particularly in America (Moye and Miles, 1988; Jones and Estes, 1995), and as such knowledge of bacteria involved in methylcarbamate degradation is of use.

Using the PCR detection method to screen oxamyl-degrading bacteria for the presence of the methylcarbamate degradation gene (*mcd*) resulted in the visualisation of *mcd* gene sized bands under gel electrophoresis in some, but not all, of the isolates tested (Chapter 3). Genes involved in carbamate degradation appear to be diverse in microbial populations, as the *mcd* gene has frequently been found to be absent from methylcarbamate degrading bacteria (Parekh *et al.*, 1995; Feng *et al.*, 1997; Desaint *et al.*, 2000). As such, a quantitative PCR assay designed to measure the frequency of the *mcd* gene within soils would be unlikely to give an accurate indication of a soil's potential for enhanced degradation. Further identification of the genes involved in methlycarbamate hydrolysis would help to make possible what could potentially be a very useful assay. As with the bacterial isolates themselves, the *mcd* gene, that has been identified in bacteria capable of

degrading a range of methylcarbamates, including carbofuran, carbaryl, aldicarb, bendiocarb and now oxamyl (Tomasek and Karns, 1989; Topp *et al.*, 1993; Chapter 3), could be used in bioremediation research to increase the substrate range of native bacteria (Chapalamadugu and Chaudhry, 1992; Top *et al.*, 2002).

Chapter 4 of this thesis details an investigation into the use of the enrichment culture method as a possible predictive tool for enhanced degradation. Although promising results were obtained, the method was shown to be less reliable in low pH soils. This method may be useful in the short-term, after further work has been carried out to better establish the pH range over which the method is reliable. However, the method is not quick (taking approximately 30 days), and also requires the use of an expensive HPLC and a large amount of incubator space. A far more suitable and quicker method would be the quantitative PCR assay mentioned above. It would therefore seem that the logical next step is to further investigate the bacteria and genes involved in enhanced degradation of methylcarbamates in the field and the factors affecting their activity, such as soil pH. In the future, it may be possible to carry out a range of diagnostic molecular tests on field soils. For example, if a farmer is planning to grow potatoes, a suite of tests could be carried out to determine the PCN population and dominant species in the field, the presence of particular disease causing viruses and fungi, and also the potential for enhanced degradation of a nematicide based on the frequency of specific degradation genes within the sample. However, until this is available the enrichment culture method may be useful for estimating a soil's potential for enhanced degradation (Chapter 4).

5.3 FURTHER WORK

Chapters 2, 3 and 4 provide clear evidence of the potential for enhanced degradation of the carbamate nematicides aldicarb and oxamyl, particularly oxamyl, within UK soils and the presence of adapted bacteria capable of oxamyl mineralisation within soils across the

country. As such, an assay designed to predict the occurrence of enhanced degradation within the field would be of use. Chapters 3 and 4 investigated this; however work is required to further develop the assays.

- The results from Chapter 4 suggest the enrichment culture method may be a suitable diagnostic assay in the short-term. However, the method was found to be less reliable in low-pH soils. Further work is required to establish a pH cut-off point for the method, *i.e.*, a point at which the pH of the soil might prevent the enhanced degradation observed in liquid enrichment from occurring in the field. This would require further investigation in the laboratory using soils that covered a range of pHs and also possible validation of the method in the field as laboratory degradation data are not necessarily transferable to the field.

- The use of a quantitative PCR method to determine the frequency of methylcarbamate degradation genes within a field seems the most suitable method for a diagnostic assay so far. The first step to developing this method requires the identification and sequencing of methylcarbamate-hydrolase genes from the oxamyl-degrading bacteria detailed in Chapter 3. Isolation of more oxamyl-degrading strains may also be necessary in order to identify as many genes as possible. It may also prove useful to investigate the ability of the oxamyl-degrading isolates to degrade other methylcarbamate pesticides, such as aldicarb and carbofuran.

In addition to predicting enhanced degradation, it may also be possible to prevent it from occurring by alternating between nematicides from different chemical groups. Chapter 2 demonstrated the greater persistence of the organophosphate fosthiazate in comparison to the carbamates oxamyl and aldicarb.

- Further work to establish whether fosthiazate is susceptible to cross-enhanced degradation in soils showing enhanced degradation of oxamyl and aldicarb would enable better advice to be given regarding rotation between the organophosphate and the two carbamates.

- It may also be of use to investigate the preventative effects of chemical rotation by carrying out a long-term study comparing the development of enhanced degradation in soils in which chemical rotation between methylcarbamate and organophosphate pesticides has been practised with soils in which only one chemical type is used.

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7. APPENDIX

Table 7.1: Concentration of oxamyl remaining (mg kg^{-1}) at time intervals (days) after application of the 1st, 2nd and 3rd applications of oxamyl. Results for duplicate replicates are displayed.

	1st application						2nd application						3rd application					
Soil	Day 0	5	10	15	20	25	0	5	10	15	21	25	0	5	11	14	20	25
2A	0.8	0.39	0.92	0.49	0.074	0	2.84	0.59	0.13	0	0	0	1.32	0.13	0	0	0	0
2B	1.55	0.88	0.58	0.38	0.095	0	2.85	0.99	0.28	0.07	0	0	1.61	0.14	0	0	0	0
3A	6.96	1.66	0.6	0.4	0.11	0	0.91	0.54	0.15	0.09	0.08	0	2.56	0.19	0.09	0.1	0.07	0
3B	7.21	1.05	0.82	0.4	0.095	0	1.14	0.37	0.13	0.09	0.07	0.07	1.74	2.09	0.11	0.09	0.07	0
4A	2.27	0.75	0.34	0.13	0	0	2.17	0.15	0	0	0	0	0.59	0.08	0	0	0	0
4B	1.37	0.69	0.43	0.14	0	0	2.29	0.11	0	0	0	0	0.7	0.08	0	0	0	0
5A	0.6	1.04	1.04	0.83	0.25	0.26	1.07	2.38	0.5	0	0	0	1.22	0.36	0.07	0	0	0
5B	0.44	0.88	0.54	0.56	0.26	0.25	2	1.04	0.74	0	0	0	1.81	0.45	0.07	0	0	0
7A	1.08	1.72	0.61	0.26	0.05	0	1.47	0.271	0.08	0	0	0	1.02	0.13	0	0	0	0
7B	1.8	0.47	0.67	0.24	0.06	0	1.16	0.29	0.08	0	0	0	1.09	0.12	0.07	0	0	0
8A	ND	2.92	0.36	0.1	0.09	0	2.54	0.85	0.55	0.29	0.21	0.14	2.25	1.0	0.7	0.08	0.16	0
8B	ND	2.54	0.76	0.34	0	0	4.37	1.11	0.25	0.32	0.2	0.13	2.59	0.83	0.3	0.22	0.09	0
9A	1.28	0.62	0.32	0.11	0.05	0	1.29	0.23	0.09	0	0	0	1.26	0.11	0.08	0	0	0
9B	1.28	0.55	0.32	0.094	0.06	0	2.01	0.27	0.11	0	0	0.09	1.04	0.14	0.09	0.11	0	0

Table 7.1 Continued

	1st application						2nd application						3rd application					
Soil	**Day 0**	**5**	**10**	**15**	**20**	**25**	**0**	**5**	**10**	**15**	**21**	**25**	**0**	**5**	**11**	**14**	**20**	**25**
10A	7.34	0.2	0	0	0	0	2.17	0	0	0	0	0	1.19	0	0	0	0	0
10B	3.5	0.17	0	0	0	0	2.01	0.07	0	0	0	0	1.05	0	0	0	0	0
11A	0.62	0.66	0.88	0.52	0.67	0.45	1.64	1.87	1.96	1.37	1.18	0.21	1.55	2.81	0.8	0.64	0.36	0.22
11B	1.39	0.63	0.84	0.6	0.26	0.44	2.29	1.6	1.68	0.91	1.04	0.34	2.19	1.60	0.9	0.53	0.23	0.15
12A	4.12	0.71	0.62	0.21	0.06	0	1.92	0.2	0.07	0	0	0	0.73	0.08	0.08	0	0	0
12B	4.08	0.91	0.67	0.28	0.05	0	0.91	1.28	0.08	0	0	0	1.19	0.11	0.07	0	0	0

Table 7.2: Concentration of fosthiazate (mg kg^{-1}) remaining at time intervals (days) after application of the 1st, 2nd and 3rd applicationss of full rate fosthiazate. Results for duplicate replicates are displayed.

	1st application						2nd application						3rd application					
Soil	**Day 0**	**5**	**10**	**15**	**20**	**25**	**0**	**5**	**10**	**15**	**21**	**25**	**0**	**5**	**11**	**14**	**20**	**25**
1A	0.53	0.42	0.46	0.59	0.54	0.76	1.47	2.05	1.77	1.78	1.57	1.78	2.69	2.69	2.95	2.58	2.47	2.41
1B	0.95	0.47	0.21	0.29	0.7	0.81	4.61	2.34	2.52	1.99	1.3	1.4	2.2	2.7	2.53	2.66	2.34	2.52
2A	0.67	0.76	0.58	0.56	0.46	0.34	1.5	1.36	1.19	1.21	1.34	1.13	1.72	1.84	1.86	1.71	1.58	1.5
2B	1.7	0.5	0.45	0.47	0.3	0.29	1.18	1.74	1.24	1.44	1.4	0.96	2.24	2.01	1.89	2.01	1.69	1.39
3A	0.32	0.58	0.35	0.27	0.26	0.19	1.24	1.11	0.75	0.6	0.63	0.32	1.27	1.14	0.8	0.63	0.49	0.4
3B	0.39	0.52	0.49	0.39	0.26	0.18	1.31	1.14	0.89	0.73	0.53	0.38	1.36	1.21	0.86	0.68	0.46	0.34
4A	0.64	1.4	0.49	0.29	0.28	0.43	1.88	2.66	1.26	0.83	0.82	0.84	1.74	1.67	1.59	1.53	1.43	1.16
4B	0.58	0.65	0.38	0.31	0.58	0.24	2.09	1.89	1.32	1	0.94	0.86	1.65	1.87	1.62	1.69	1.44	1.22
5A	0.64	0.19	0.19	0.37	0.28	0.22	0.87	1.39	2	0.6	1	0.74	3.41	2.44	1.22	1.18	0.94	0.91
5B	0.76	0.31	0.4	0.38	0.63	0.23	1.26	1.11	1.13	1.02	0.98	0.77	2.9	2.05	1.31	1.18	1.0	0.94

Table 7.2 Continued

	1st application						2nd application						3rd application					
Soil	**Day 0**	**5**	**10**	**15**	**20**	**25**	**0**	**5**	**10**	**15**	**21**	**25**	**0**	**5**	**11**	**14**	**20**	**25**
6A	1.58	0.27	0.33	0.34	0.24	0.32	1.6	1.36	0.91	1.49	0.92	0.81	1.96	1.65	1.5	1.39	1.11	1.38
6B	3.39	0.17	0.31	0.21	0.29	0.6	2.74	1.15	1.35	1.54	0.87	0.81	3	1.49	1.76	1.38	1.06	1.1
7A	1.55	0.68	0.7	0.7	0.64	0.76	1.88	1.76	1.6	1.74	1.54	1.45	2.23	2.38	2.1	2.15	1.85	1.85
7B	0.95	0.63	0.47	0.52	0.55	0.62	1.81	1.46	1.5	1.69	1.53	1.64	2.85	2.66	1.51	2.4	1.7	1.84
8A	0.84	0.76	0.37	0.5	0.58	0.59	0.91	1.5	1.48	1.57	1.58	1.5	2.35	2.3	2.19	2.27	1.93	2.0
8B	1.43	0.27	0.47	0.8	0.52	0.52	2.02	1.85	1.51	1.51	1.55	1.35	2.29	2.13	2.17	2	1.88	1.91
9A	0.18	0.26	0.32	0.38	0.35	0.39	2.96	1.39	1.26	1.3	1.45	1.12	2.19	2.05	2.05	1.92	1.65	1.67
9B	0.7	0.29	0.44	0.46	0.41	0.64	2.27	1.37	1.28	1.34	1.47	1.18	2.07	2.04	2.05	1.97	1.47	1.69
10A	0.89	0.59	0.35	0.51	0.71	0.45	1.81	1.48	1.48	1.28	1.36	1.3	2.08	2.03	2.17	2.17	1.95	1.86
10B	1.05	0.77	0.59	0.51	0.64	0.5	1.48	1.38	1.6	1.76	1.43	1.44	2.02	2.08	2.31	2.22	1.94	1.79

Table 7.3: Concentration (mg kg^{-1}) of the total toxic residues of aldicarb and its oxidation products remaining at time points (days) after application of the 1st, 2nd and 3rd applications of aldicarb. Results for duplicate replicates are displayed.

	1st application						2nd application						3rd application					
Soil	***Day0***	***5***	***10***	***15***	***20***	***25***	***0***	***5***	***10***	***15***	***21***	***25***	***0***	***5***	***11***	***14***	***20***	***25***
1A	1.248	1.189	1.399	1.561	1.041	1.092	2.286	2.163	2.923	2.079	2.273	1.820	3.113	2.784	2.592	2.384	2.022	1.794
1B	2.150	1.683	1.426	1.413	0.998	1.300	2.383	2.633	1.987	1.980	1.660	1.680	2.975	2.680	2.489	2.251	1.891	1.668
2A	2.807	2.293	2.301	1.521	1.803	1.226	5.588	2.984	2.095	1.676	1.516	2.840	3.900	3.228	4.411	3.845	4.068	3.540
2B	0.897	1.436	1.517	1.896	0.657	0.975	2.839	2.579	2.501	1.541	1.651	2.617	4.043	6.507	3.997	4.127	3.944	3.385
3A	5.764	8.192	2.997	3.027	1.509	1.317	7.385	3.964	4.045	1.944	3.582	2.915	9.364	9.813	5.007	2.305	3.489	3.077
3B	9.557	5.718	3.572	2.436	1.623	1.212	7.476	5.606	3.106	2.959	3.373	3.414	9.401	7.716	4.258	4.015	3.590	2.443
4A	3.294	1.040	0.613	0.358	0.038	0.145	1.524	0.233	0.033	0.021	0.043	0.125	1.023	0.030	0	0	0	0
4B	3.347	0.100	0.715	0.327	0.024	0.014	1.008	0.111	0.019	0.023	0.023	0	0.924	0.041	0	0	0	0

Table 7.3 Continued

	1st application						2nd application						3rd application					
Soil	***Day 0***	***5***	***10***	***15***	***20***	***25***	***0***	***5***	***10***	***15***	***21***	***25***	***0***	***5***	***11***	***14***	***20***	***25***
5A	0.740	2.894	3.198	2.211	2.149	2.019	2.267	2.720	2.805	3.601	2.871	3.145	4.247	4.454	4.167	4.873	4.184	4.004
5B	1.660	1.918	2.111	1.273	2.925	1.168	2.439	1.967	2.758	3.401	3.214	3.188	3.671	4.005	3.835	4.131	3.773	3.392
6A	0.822	0.811	1.713	0.824	0.358	0.619	1.911	0.742	0.244	0.248	0.101	0.149	1.120	0.165	0.015	0.027	0.202	0.155
6B	1.927	0.605	1.288	0.855	0.475	0.665	2.443	1.133	1.200	0.680	0.739	0.053	1.216	0.041	0	0.020	0.016	0.029
7A	2.183	0.832	1.067	0.731	0.323	0.300	1.289	0.707	0.225	0.024	0.026	0.148	1.141	0.213	0	0.179	0.151	0
7B	1.067	3.210	1.113	0.684	0.32	0.414	1.876	0.694	0.199	0.033	0.029	0	1.023	0.066	0	0	0	0
8A	5.382	1.089	1.876	1.168	1.142	1.160	1.723	1.872	1.555	1.349	1.155	0.975	1.948	1.341	1.078	0.862	0.628	0.370
8B	3.041	1.468	2.443	1.571	1.187	1.126	1.661	1.920	1.533	1.548	1.012	0.975	1.938	1.560	1.189	0.914	0.727	0.540
9A	9.944	0.666	1.301	0.679	0.490	0.415	1.756	0.652	0.252	0.100	0	0.121	1.043	0	0.186	0	0.135	0
9B	5.350	1.177	1.190	0.735	0.553	0.385	1.757	0.824	0.309	0.027	0	0	0.893	0.012	0	0	0	0
10A	3.243	1.513	1.066	0.747	0.349	0.262	2.057	0.537	0.158	0.013	0.016	0.144	1.006	0	0.134	0.165	0.129	0.132
10B	4.167	1.368	0.975	0.633	0.369	0.327	1.598	0.581	0.047	0.012	0	0	1.084	0	0	0	0	0
11A	5.516	0.285	0.165	0	0	0.308	1.206	0.368	0.19	0.151	0.071	0	0.324	0.232	0.285	0.220	0	0.241
11B	1.971	0.421	0.151	0	0	0.163	1.414	0.238	0.241	0.110	0.087	0.203	0.971	0.178	0	0	0.104	0
12A	9.211	1.848	1.140	1.047	0.747	0.723	1.480	0.885	0	0.221	0.05	0.012	1.159	0.279	0.210	0	0.121	0
12B	7.884	0.848	1.288	0.685	0.842	0.906	1.215	0.905	0.205	0.097	0.045	0.050	0.713	0.086	0.018	0	0	0
13A	0.761	0.671	0.518	0.111	0	0.146	0.744	0.229	0.031	0.164	0	2.544	0.963	0.125	0.143	0.132	0	0.133
13B	-	0.873	0.562	0.111	0	0.058	1.579	0.085	0.03	0.024	0	3.117	0.815	0.021	0	0	0	0
14A	1.135	0.830	0.984	0.460	0.174	0.419	1.193	0.566	0.091	0.129	0	0.159	0.521	0.305	0.125	0	0.139	0
14B	1.436	0.815	0.841	0.424	0.137	0.213	1.053	0.459	0.096	0.148	0.022	0	0.615	0.066	0.002	0	0	0
15A	1.011	0.907	0.546	0.383	0.295	0.253	1.601	0.459	0.168	0.071	0.019	0	1.155	0.181	0	0	0.127	0
15B	2.38	1.976	0.798	0.408	0.149	0.049	1.539	0.401	0.090	0.068	0.021	0	1.07	0.062	0	0	0	0

Table 7.4: Degradation of oxamyl (mg kg^{-1}) in test culture with OX10 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Soil and isolate	***Day 6***	***Day 13***	***Day 20***
Soil 10 1S3/1	12.567	9.385	7.065
Soil 10 1S3/2	12.803	9.318	7.043
Soil 10 1S3/3	12.371	9.070	6.721
Soil 10 1S3/4	11.748	8.749	6.828
Soil 10 1S3/5	12.091	8.840	6.749
Soil 10 1S3/6	11.836	8.770	6.484
Soil 10 1S3/7	12.264	9.045	6.827
Soil 10 1S3/8	12.026	9.050	6.704
Soil 10 1S3/9	9.065	2.611	0.000
Soil 10 1S3/10	12.166	8.855	6.673
Soil 10 1S3/11	12.010	9.091	6.748
Soil 10 1S3/12	12.050	9.083	6.891
Soil 10 1S3/13	12.074	8.939	6.757
Soil 10 1S3/14	12.037	8.927	6.663
Soil 10 1S3/15	0.575	0	0
Soil 10 1S3/15A	0	0	0
Soil 10 2S3/1	9.028	2.295	0
Soil 10 2S3/2	12.435	9.121	7.025
Soil 10 2S3/3	12.246	9.307	6.928
Soil 10 2S3/4	11.879	9.030	6.630
Soil 10 2S3/5	12.326	9.192	6.849
Soil 10 2S3/6	12.344	9.164	6.800
Soil 10 2S3/7	12.263	9.007	6.782
Soil 10 2S3/8	12.350	9.123	6.937
Soil 10 2S3/9	12.406	9.206	6.825
Soil 10 2S3/10	12.151	9.118	6.633
Soil 10 2S3/11	12.128	9.060	6.740
Soil 10 2S3/12	0	0	0
Soil 10 2S3/13	10.607	4.590	0
Soil 10 2S3/14	12.127	8.825	6.782
Soil 10 2S3/15	12.040	8.936	6.655
Soil 10 2S3/16	12.335	9.211	6.909
Soil 10 2S3/17	12.673	9.430	6.999
Soil 10 2S3/18	12.164	9.048	6.685

Table 7.5: Degradation rate of oxamyl (mg kg^{-1}) in test culture with OX7 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Soil and isolate	*Day 6*	*Day 9*	*Day 15*	*Day 20*
Soil 7 1S3/1	11.300	9.797	7.501	5.899
Soil 7 2S3/1	11.623	10.072	7.765	5.981
Soil 7 3S3/1	0.081	0.000	0.000	0.000
Soil 7 3S3/2	0.245	0.000	0.000	0.000
Soil 7 3S3/3	11.224	9.699	7.671	5.929
Soil 7 3S3/4	11.325	9.831	7.539	6.015
Soil 7 3S3/5	0.000	0.000	0.000	0.000
Soil 7 3S3/6	11.112	9.631	7.797	5.848
Soil 7 3S3/7	10.993	9.558	7.425	5.948

Table 7.6: Degradation rate of oxamyl (mg kg^{-1}) in test culture with OX4 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Soil and isolate	*Day 6*	*Day 9*	*Day 15*	*Day 20*
Soil 4 1S3/1	10.232	8.697	3.548	0.000
Soil 4 1S3/2	11.166	9.748	7.371	6.021
Soil 4 1S3/3	10.842	9.566	7.286	5.751
Soil 4 1S3/4	11.057	9.594	7.354	6.637
Soil 4 1S3/5	0.000	0.000	0.000	0.000
Soil 4 2S3/1	0.000	0.000	0.000	0.000
Soil 4 2S3/2	10.873	9.491	6.986	5.769
Soil 4 2S3/3	0.000	0.000	0.000	0.000
Soil 4 2S3/4	11.036	9.486	7.234	5.797
Soil 4 2S3/5	11.128	9.620	7.390	5.879
Soil 4 2S3/6	10.980	9.618	7.427	6.064
Soil 4 2S3/7	0.165	0.000	0.000	0.000
Soil 4 3S3/1	10.967	8.158	0.269	0.000
Soil 4 3S3/2	11.285	9.965	7.399	5.954
Soil 4 3S3/3	9.304	4.813	0.289	0.000
Soil 4 3S3/4	10.983	9.633	7.531	5.774
Soil 4 3S3/5	10.954	9.729	7.189	5.772
Soil 4 3S3/6	10.922	9.632	7.212	5.780
Soil 4 3S3/7	0.000	0.000	0.000	0.000
Soil 4 3S3/8	11.158	9.708	7.252	5.721
Soil 4 3S3/9	11.151	9.486	7.164	5.669

Table 7.7: Degradation of oxamyl (mg kg^{-1}) in test cultures with soil OX2 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Isolate	*Day 3*	*10*	*20*
Soil 2 21S3/1	13.518	9.917	5.982
Soil 2 21S3/2	10.988	0.680	0
Soil 2 21S3/3	1.839	0	0
Soil 2 21S3/5	9.679	2.144	0.141
Soil 2 21S3/6	0	0	0
Soil 2 22S3/1	0.466	0	0
Soil 2 22S3/4	12.356	1.780	0
Soil 2 22S3/5	12.275	10.166	6.018
Soil 2 22S3/6	12.338	9.434	6.023
Soil 2 23S3/3	1.956	0	0
Soil 2 23S3/4	13.039	10.137	6.210
Soil 2 23S3/5	12.615	9.752	6.098
Soil 2 23S3/6	6.082	0	0
Soil 2 23S3/7	9.303	0.558	0

Table 7.8: Degradation of oxamyl (mg kg^{-1}) in test cultures with soil OX9 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Isolate	*Day 3*	*10*	*20*
Soil 9 91S3/1	13.576	10.356	6.275
Soil 9 91S3/2	13.032	9.836	5.205
Soil 9 91S3/3	13.119	9.890	7.904
Soil 9 91S3/4	0.163	0	0
Soil 9 91S3/6	3.817	0	0
Soil 9 91S3/7	13.321	10.052	6.132
Soil 9 92S3/1	12.382	9.659	6.197
Soil 9 92S3/2	0	0	0
Soil 9 92S3/3	12.581	9.348	5.758
Soil 9 92S3/4	13.171	1.389	0
Soil 9 92S3/5	3.404	0	0
Soil 9 92S3/7	12.741	9.227	5.678
Soil 9 92S3/9	12.461	9.619	5.919
Soil 9 93S3/1	12.656	9.348	5.695
Soil 9 93S3/2	12.492	9.552	5.917
Soil 9 93S3/3	12.861	9.532	5.945
Soil 9 93S3/4	12.599	9.235	5.955
Soil 9 93S3/5	12.938	9.589	6.130
Soil 9 93S3/6	12.984	9.475	5.977
Soil 9 93S3/7	12.828	9.502	5.861
Soil 9 93S3/8	13.491	9.721	6.053
Soil 9 93S3/9	13.125	9.688	5.816
Soil 9 93S3/10	13.376	10.089	6.340
Soil 9 93S3/11	13.232	9.978	6.136
Soil 9 93S3/12	13.015	9.740	6.067

Table 7.9: Degradation of oxamyl (mg kg^{-1}) in test cultures with soil OX5 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Isolate	*Day 5*	*11*	*20*
Soil 5 51S3/1	13.002	10.010	6.407
Soil 5 51S3/2	13.165	9.837	6.584
Soil 5 51S3/3	12.989	9.593	6.555
Soil 5 51S3/4	12.835	9.843	6.187
Soil 5 51S3/5	13.650	9.860	6.334
Soil 5 51S3/6	0.202	0	0
Soil 5 51S3/7	13.139	10.107	6.651
Soil 5 51S3/8	12.960	9.862	6.244
Soil 5 51S3/9	12.979	9.883	6.438
Soil 5 51S3/10	12.794	9.742	6.516
Soil 5 51S3/11	13.178	9.696	6.434
Soil 5 52S3/1	13.289	9.803	6.614
Soil 5 52S3/2	0.380	0	0
Soil 5 52S3/3	12.512	9.855	6.446
Soil 5 52S3/4	12.908	9.764	6.531
Soil 5 52S3/5	12.972	9.923	6.384
Soil 5 52S3/6	13.032	9.953	6.654
Soil 5 52S3/7	12.998	9.728	6.409
Soil 5 52S3/8	0.382	0	0
Soil 5 52S3/9	6.887	0.589	0
Soil 5 52S3/10	0.258	0	0
Soil 5 53S3/1	12.660	9.159	6.077
Soil 5 53S3/2	12.843	9.251	6.016
Soil 5 53S3/3	12.953	9.519	6.249
Soil 5 53S3/4	12.687	9.424	6.271
Soil 5 53S3/5	12.871	9.571	6.124
Soil 5 53S3/6	13.142	9.653	6.505
Soil 5 53S3/7	12.908	9.499	6.178
Soil 5 53S3/8	13.346	9.849	6.423
Soil 5 53S3/9	12.562	9.542	6.385
Soil 5 53S3/10	12.762	9.601	6.272
Soil 5 53S3/11	12.529	9.457	6.197

Table 7.10: Degradation of oxamyl (mg kg^{-1}) in test cultures with soil OX12 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Isolate	*Day 5*	*11*	*20*
Soil 12 121S3/1	12.939	9.918	6.272
Soil 12 121S3/2	13.056	9.735	6.113
Soil 12 121S3/3	12.505	9.758	6.138
Soil 12 121S3/4	12.605	9.860	6.240
Soil 12 121S3/5	12.621	9.956	6.301
Soil 12 121S3/6	12.347	9.634	5.992
Soil 12 121S3/7	12.368	9.726	6.201
Soil 12 121S3/8	12.424	9.794	6.184
Soil 12 121S3/9	0	0	0
Soil 12 121S3/10	0	0	0
Soil 12 123S3/1	12.716	9.709	6.031
Soil 12 123S3/2	12.244	9.514	6.188
Soil 12 123S3/3	12.151	9.494	6.037
Soil 12 123S3/4	12.380	9.495	6.141
Soil 12 123S3/5	12.504	9.676	6.329
Soil 12 123S3/6	12.804	9.784	6.366
Soil 12 123S3/7	12.512	9.268	6.398
Soil 12 123S3/8	12.215	9.577	6.355
Soil 12 123S3/9	12.278	9.489	6.341
Soil 12 123S3/10	11.553	9.566	6.103
	Day 0	*11*	*20*
Soil 12 122S3/1	14.544	9.742	6.107
Soil 12 122S3/2	14.621	10.320	6.299
Soil 12 122S3/3	14.765	9.908	6.483
Soil 12 122S3/4	14.787	9.735	6.216
Soil 12 122S3/5	14.454	9.440	6.051
Soil 12 122S3/6	15.290	9.565	6.282
Soil 12 122S3/7	14.507	9.417	6.274
Soil 12 122S3/8	14.530	9.458	6.108
Soil 12 122S3/9	15.495	0.399	0
Soil 12 122S3/10	14.315	9.217	5.992
Soil 12 122S3/11	14.328	8.853	5.717

Table 7.11: Degradation of oxamyl (mg kg^{-1}) in test cultures with soil OX3 isolates. D0 oxamyl concentration was approximately 15 mg kg^{-1}

Isolate	*Day 0*	*10*	*20*
Soil 3 31S3/1	15.842	10.232	6.559
Soil 3 31S3/2	14.968	9.764	6.344
Soil 3 31S3/3	14.509	9.356	6.235
Soil 3 31S3/4	15.089	10.914	6.797
Soil 3 31S3/5	14.954	10.042	6.714
Soil 3 31S3/6	14.857	9.748	6.142
Soil 3 31S3/7	14.835	9.899	6.361
Soil 3 31S3/8	14.857	0.435	0
Soil 3 31S3/9	14.833	9.736	6.297
Soil 3 31S3/10	14.809	9.948	6.528
Soil 3 31S3/11	14.923	9.9543	6.254
Soil 3 32S3/2	14.906	9.525	6.193
Soil 3 32S3/3	14.961	9.496	6.035
Soil 3 32S3/6	14.918	2.607	0.145
Soil 3 32S3/7	14.789	9.413	6.262
Soil 3 32S3/8	15.120	9.810	6.480
Soil 3 32S3/9	14.862	9.692	6.333
Soil 3 32S3/10	14.759	0.128	0
Soil 3 32S3/11	14.793	9.735	6.280
Soil 3 32S3/12	14.775	9.497	6.466
Soil 3 32S3/13	15.191	9.979	6.702
Soil 3 33S3/1	15.264	10.707	6.118
Soil 3 33S3/2	14.942	10.330	5.941
Soil 3 33S3/3	15.081	10.544	6.346
Soil 3 33S3/4	14.839	10.680	6.346
Soil 3 33S3/6	14.619	10.024	5.919
Soil 3 33S3/7	14.956	10.437	6.066
Soil 3 33S3/8	15.035	10.224	5.855
Soil 3 33S3/9	15.122	10.407	5.881
Soil 3 33S3/10	14.679	0	0
Soil 3 33S3/11	14.827	10.595	5.976

Table 7.12: Degradation of oxamyl (mg kg^{-1}) in previously treated (T) and previously untreated (U) soils in the Incubation Study.

Soil	Replicate	Day 5	10	15	20	25	30
1T	A	1.637	1.281	0.851	0.233	0.19	0.145
	B	1.409	1.217	0.74	0.18	0.182	0.126
	C	1.586	1.251	0.615	0.447	0.31	0.167
1U	A	1.282	2.515	0.974	0.638	0.521	0.42
	B	1.541	1.001	0.796	0.606	0.427	0.282
	C	3.976	1.213	0.59	0.581	0.48	0.438
2T	A	1.508	0.607	0.802	0	0	0
	B	1.319	0.644	0.896	0	0	0
	C	1.547	0.648	0.211	0	0	0
2U	A	1.117	0.925	0.194	0.2469	0	0
	B	1.66	0.801	0.224	0.31	0.151	0
	C	2.03	0.829	0.582	0.213	0.198	0
3T	A	1.311	0.583	0.285	0.0921	0	0
	B	1.778	0.895	0.344	0.0868	0	0
	C	1.537	0.852	0.354	0.126	0	0
3U	A	1.627	1.2	0.901	0.524	0.322	0.288
	B	2.531	1.09	0.743	0.596	0.26	0.195
	C	0.62	1.078	0.614	0.387	0.265	0.191
4T	A	1.591	1.399	0.79	0.156	0	0
	B	1.313	1.067	0.611	0.178	0	0
	C	2.065	1.965	0.687	0.163	0	0
4U	A	2.412	1.329	0.602	0.151	0	0
	B	2.099	1.097	0.594	0.147	0.122	0
	C	1.88	1.497	0.699	0.155	0.111	0
5T	A	2.262	1.546	0.64	0.15	0	0
	B	1.519	1.238	0.609	0.193	0	0
	C	1.862	1.14	0.576	0	0	0
5U	A	2.012	1.336	0.796	0.22	0	0
	B	1.995	1.235	0.946	0.259	0.143	0
	C	1.656	1.184	0.829	0.32	0.163	0.124
6T	A	1.355	0.467	0.171	0	0	0
	B	1.313	0.452	0.154	0	0	0
	C	1.091	0.374	0.181	0	0	0
6U	A	1.478	1.273	0.613	0.265	0.193	0.119
	B	1.671	1.172	0.616	0.24	0.169	0.153
	C	1.818	1.541	0.693	0.244	0.159	0
7T	A	1.544	0.57	0.282	0.097	0.014	0
	B	1.476	0.597	0.26	0.091	0	0
	C	1.284	0.554	0.239	0.113	0	0
7U	A	1.975	1.434	1.256	0.969	0.745	0.677
	B	1.987	1.588	1.314	1.209	0.889	0.761
	C	2.092	1.549	1.321	1.124	0.762	0.697
8T	A	0.417	0.229	0.175	0	0	0
	B	0.374	0.134	0	0	0	0
	C	0.334	0	0	0	0	0

Table 7.12 continued: Degradation of oxamyl (mg kg^{-1}) in previously treated (T) and previously untreated (U) soils in the Incubation Study.

Soil	Replicate	Day 5	10	15	20	25	30	35	40
8U	A	1.437	1.308	1.086	0.91	0.757	0.678		
	B	2.141	2.194	1.473	1.061	0.838	0.787		
	C	1.957	1.925	1.235	0.998	0.77	0.848		
9T	A	1.561	0.677	0.244	0.0837	0	0		
	B	1.422	0.799	0.211	0.0813	0	0		
	C	1.316	0.713	0.188	0	0	0		
9U	A	1.693	1.394	1.044	0.968	0.779	0.765		
	B	3.311	1.242	1.575	1.342	0.945	0.841		
	C	1.775	1.621	1.317	1.11	1.04	0.828		
10T	A	2.708	1.691	1.856	1.725	1.689	1.791	1.582	1.538
	B	2.473	3.085	1.949	2.294	2.885	2.012	1.85	2.203
	C	3.12	1.923	1.65	1.712	1.843	1.769	1.707	1.808
10U	A	1.736	2.47	1.544	1.317	1.33	1.237	0.918	0.984
	B	1.96	4.027	1.679	1.396	1.276	1.356	1.113	1.149
	C	1.77	1.566	1.328	1.063	1.041	0.967	0.804	0.757
11T	A	2.145	1.567	1.667	1.688	1.494	1.211	0.855	0.752
	B	1.66	1.722	1.576	1.092	1.143	1.211	0.761	0.804
	C	2.4	3.222	1.601	1.272	1.171	1.383	0.831	0.762
11U	A	1.896	2.852	1.695	1.433	1.388	1.507	0.93	0.964
	B	2.027	2.08	1.942	1.336	1.417	1.544	1.11	1.088
	C	2.017	1.983	1.67	1.381	1.307	1.527	0.982	0.878
12T	A	3.155	2.565	2.117	1.98	1.801	2.637	1.616	1.879
	B	1.844	2.061	2.029	2.076	2.029	2.141	1.395	1.549
	C	1.906	2.13	2.049	1.686	1.761	2.019	1.438	1.686
12U	A	1.844	2.015	2.204	1.053	1.212	1.279	0.729	0.703
	B	2.348	1.988	1.474	1.069	1.234	1.059	0.846	0.72
	C	1.849	2.055	1.698	1.087	1.113	1.113	0.731	0.686
13T	A	2.725	1.705	1.971	1.552	1.265	1.406	0.886	1.026
	B	2.52	2.672	2.467	1.745	1.454	1.491	0.922	0.846
	C	2.336	1.625	1.461	1.151	1.118	1.273	0.886	0.716
13U	A	1.76	1.585	0.445	0.082	0.13	0.11	0	0
	B	1.898	1.602	0.439	0.023	0	0	0	0
	C	0.832	1.274	0.418	0.119	0	0	0	0
14T	A	2.33	1.837	1.622	2.436	1.913	2.81	1.538	1.479
	B	2.14	2.975	1.97	2.189	2.235	2.231	1.66	1.68
	C	1.708	2.196	1.845	1.444	1.683	1.87	1.423	1.483
14U	A	1.403	2.185	1.465	1.443	1.361	1.168	0.951	0.794
	B	1.927	1.748	1.702	1.39	1.225	1.079	0.921	0.783
	C	2.189	1.685	1.672	1.18	1.2	1.007	0.842	0.861
15T	A	2.519	2.448	2.431	2.168	2.108	1.975	1.77	2.082
	B	2.925	2.527	2.175	2.437	2.176	2.016	2.012	2.276
	C	2.397	2.477	2.239	2.387	2.164	2.225	2.042	2.061
15U	A	1.593	1.522	1.557	1.82	1.526	1.522	1.311	1.269
	B	1.853	2.397	2.036	2.042	1.758	1.914	1.402	1.473
	C	2.182	2.34	1.796	2.117	1.842	2.457	1.329	1.382

Table 7.12 continued: Degradation of oxamyl (mg kg^{-1}) in previously treated (T) and previously untreated (U) soils in the Incubation Study.

Soil	Replicate	Day 5	10	15	20	25	30	35	40
16T	A	2.455		1.799		1.546	1.452	1.075	0.897
	B	2.458	2.759	2.228	2.51	1.621	1.376	1.212	0.98
	C	3.161	3.479	1.603	1.863	1.5	1.453	1.242	1.002
16U	A	2.581	2.014	2	2.191	1.651	1.501	1.704	1.382
	B	1.843	1.856	1.836	1.498	1.654	1.397	1.364	1.417
	C	2.416	1.771	1.841	1.957	1.443	1.635	1.206	1.326
17T	A	3.171	2.585	2.362	2.862	2.325	1.945	1.532	1.671
	B	2.42	2.299	2.246	2.513	1.912	1.963	1.581	1.442
	C	3.31	2.831	2.638	2.712	2.36	2.075	1.852	1.526
17U	A	1.574	2.518	1.092	0.726	0.636	0.487	0.336	0.277
	B	1.67	1.656	1.179	0.891	0.616	0.598	0.389	0.347
	C	2.246	1.312	1.117	0.965	0.857	0.749	0.508	0.437
18T	A	2.19	1.711	1.698	2.002	1.802	1.497	1.018	0.79
	B	2.304	1.674	1.49	2.031	1.368	1.247	1.043	0.726
	C	2.476	2.458	1.82	1.604	1.721	1.324	0.872	0.798
18U	A	1.743	1.42	0.793	0.266	0.242	0.158	0.052	0.051
	B	1.785	1.222	0.788	0.314	0.273	0.209	0.054	0
	C	1.966	1.748	0.77	0.383	0.22	0.169	0	0
19T	A	2.698	2.223	1.728	1.997	1.652	1.439	1.155	1.122
	B	1.849	2.108	1.814	1.962	1.798	1.518	1.138	1.082
	C	2.149	1.876	1.92	1.842	1.649	1.449	1.285	0.96
19U	A	1.717	1.694	1.674	1.377	1.016	0.841	0.661	0.655
	B	1.766	2.167	1.389	1.05	0.947	0.856	0.576	0.512
	C	1.927	1.484	1.42	1.272	1.218	0.849	0.657	0.607

Table 7.13: Oxamyl degradation (mg kg^{-1}) in previously treated (T) and previously untreated (U) soil samples in the Enrichment Culture Study.

Soil	Replicate	Day 0	5	10	15	20	25	30
1T	A	16.218	13.136	10.504	8.084	3.088	0.946	0.26
	B	16.376	12.988	10.497	8.741	5.306	3.791	2.794
	C							
1U	A	15.839	13.24	10.815	8.303	3.012	0.992	0.264
	B	16.352	13.357	10.703	8.961	6.276	5.206	3.868
	C	15.835	13.121	10.233	8.656	5.76	4.66	3.652
2T	A	16.214	13.265	8.812	3.48	0.343	0.244	0.134
	B	16.391	13.467	9.307	4.078	0.249	0	0
	C	16.615	13.759	9.264	4.327	0.348	0	0
2U	A	15.974	12.988	10.332	8.743	5.252	3.669	2.154
	B	16.494	13.477	10.745	8.648	5.219	2.63	0.985
	C	16.381	13.164	10.803	8.821	5.353	3.304	1.69
3T	A	16.267	13.197	9.387	5.058	0.904	0.287	0.109
	B	16.416	13.6	9.677	5.255	0.664	0.169	0
	C	15.981	13.049	9.174	4.604	0.439	0	0
3U	A	15.854	12.92	4.725	8.604	5.856	5.014	4.361
	B	16.165	13.51	10.706	8.764	5.997	5.177	3.969
	C	15.542	12.962	10.641	8.445	4.865	3.37	1.975
4T	A	17.213	13.703	10.979	8.971	3.871	1.685	0.663
	B	18.07	14.576	12.114	9.443	3.97	1.394	0.339
	C	15.763	12.994	10.398	8.196	3.865	1.533	0.621
4U	A	16.301	13.08	10.845	7.757	1.852	0.327	0
	B	15.895	13.137	9.677	5.636	0.388	0	0
	C	16.325	13.05	10.42	7.756	1.516	0.298	0
5T	A	16.023	13.022	10.135	8.931	7.407	2.397	0.952
	B	15.92	13.407	10.413	8.461	4.148	1.919	0.858
	C	14.916	12.593	10.078	8.471	4.426	2.529	1.422
5U	A	15.997	13.337	7.639	9.104	6.258	5.057	3.625
	B	16.185	12.792	10.154	8.475	5.469	3.772	2.777
	C	16.378	13.216	10.452	8.697	5.72	4.258	2.781
6T	A	15.889	13.056	9.863	7.311	2.828	1.401	0.723
	B	15.62	12.696	9.972	7.123	2.879	1.39	0.507
	C	16.158	12.994	9.977	7.323	2.949	1.521	1.016
6U	A	15.984	12.736	10.403	8.428	5.07	3.443	2.554
	B	15.146	12.341	10.211	8.443	5.357	3.893	2.664
	C	14.998	12.48	9.992	7.587	5.047	4.097	2.74
7T	A	15.867	13.02	9.636	8.734	2.18	1.292	0.624
	B	15.729	12.452	9.587	2.762	0	0	0
	C	15.957	12.648	9.615	7.321	3.585	2.13	1.3366
7U	A	16.173	13.127	10.347	8.191	5.577	4.704	3.386
	B	14.759	12.223	9.49	7.794	5.333	4.182	3.254
	C	15.647	13.017	10.467	8.114	5.772	4.583	3.579
8T	A	15.808	11.418	2.485	0.759	0.286	0.232	0.176
	B	15.865	12.044	0.658	0.134	0	0	0
	C	15.741	11.471	0.971	0.231	0	0	0

Table 7.13 continued: Oxamyl degradation (mg kg^{-1}) in previously treated (T) and previously untreated (U) soil samples in the Enrichment Culture Study.

Soil	Replicate	Day 0	5	10	15	20	25	30
8U	A	15.656	13.372	9.778	5.232	0.122	0	0
	B	15.209	12.229	10.772	8.701	5.923	4.645	4.15
	C	15.123	12.462	10.25	8.381	5.898	4.792	3.814
9T	A	14.237	12.715	8.434	3.76	0.627	0.284	0.193
	B	15.178	12.939	8.204	4.188	0.532	0.177	0
	C	15.266	12.428	9.25	5.312	0.823	0.239	0
9U	A	15.316	13.145	10.733	9.093	5.997	4.957	3.984
	B	15.9	12.789	10.878	8.751	5.924	4.958	3.477
	C	15.681	12.847	11.019	9.467	6.408	5.29	4.244
10T	A	15.861	13.203	11.107	10.034	7.099	6.371	4.606
	B	16.891	13.364	11.9	9.624	6.923	5.84	5.023
	C	15.498	12.871	11.009	8.768	6.729	5.892	4.805
10U	A	15.973	12.936	11.357	9.719	6.91	5.924	4.599
	B	16.146	12.815	11.306	9.966	6.798	5.987	4.826
	C	15.453	12.257	10.761	9.427	6.459	5.295	4.248
11T	A	16.317	13.312	11.049	3.331	0.338	0.323	0.207
	B	16.06	13.384	8.889	0.277	0	0	0
	C	15.589	13.492	9.265	0.335	0	0	0
11U	A	15.791	13.479	11.407	10.016	7.134	5.59	5.589
	B	15.436	13.265	11.498	10.54	7.177	5.886	5.826
	C	16.544	13.842	11.153	10.02	7.087	5.274	5.015
12T	A	15.982	12.195	10.918	9.098	6.734	5.25	4.668
	B	15.995	12.492	10.616	9.137	6.84	5.49	4.911
	C	15.79	12.714	10.942	9.44	7.27	5.759	5.029
12U	A	15.921	12.76	10.209	9.131	6.708	4.77	4.292
	B	16.256	12.751	10.192	9.515	6.323	5	4.603
	C	15.377	12.764	10.316	8.678	6.959	5.019	4.834
13T	A	15.505	12.846	10.259	3.937	0	0.269	0.124
	B	15.876	12.857	10.413	4.587	0	0	0
	C	15.682	12.782	10.382	6.463	0	0	0
13U	A	15.532	12.45	9.697	0.626	0	0	0
	B	15.708	12.411	8.07	0.184	0	0	0
	C	15.739	12.48	9.905	0.424	0	0	0
14T	A	15.418	12.947	10.597	6.707	0	0	0
	B	15.69	12.684	10.573	8.9	6.007	4.484	3.782
	C	16.258	12.827	10.508	8.993	6.6	5.283	4.411
14U	A	15.515	12.711	10.361	8.779	6.306	5.189	4.499
	B	15.438	12.428	10.247	10.426	7.009	6.082	4.91
	C	15.285	12.625	10.425	9.136	6.533	5.42	4.485
15T	A	15.737	12.848	10.769	9.066	6.814	5.654	4.962
	B	15.83	12.862	10.912	8.902	6.607	5.426	4.566
	C	14.69	12.094	10.111	8.805	6.269	5.304	4.699
15U	A	16.152	13.411	11.353	9.51	6.878	5.745	5.119
	B	15.598	12.989	10.328	8.984	6.725	5.374	4.163
	C	15.81	12.637	10.33	9.4	6.566	5.576	4.51
16T	A	15.536	12.456	9.891	8.656	6.155	5.231	3.961
	B	15.681	13.003	10.25	8.757	5.967	4.562	3.625
	C	15.002	12.687	10.084	9.163	6.427	4.284	1.804

Table 7.13 continued: Oxamyl degradation (mg kg^{-1}) in previously treated (T) and previously untreated (U) soil samples in the Enrichment Culture Study.

Soil	Replicate	Day 0	5	10	15	20	25	30
16U	A	15.203	12.219	10.23	8.035	0	0.235	0
	B	15.315	12.407	10.084	7.892	0	0	0
	C	14.559	11.811	9.579	7.978	3.632	0.948	0.207
17T	A	15.074	12.529	10.067	3.94	0	0	0
	B	15.336	12.57	10.133	4.942	0	0	0
	C	15.345	12.777	10.021	3.686	0	0	0
17U	A	15.209	12.215	9.531	7.238	0.397	0	0
	B	15.77	12.615	9.996	7.918	0	0	0
	C	15.596	13.116	10.246	8.313	0.64	0	0
18T	A	15.469	12.416	10.169	8.558	1.505	0	0
	B	15.496	12.73	10.381	8.896	6.006	4.313	2.48
	C	16.045	12.716	10.318	8.864	5.95	4.333	2.822
18U	A	15.585	12.29	9.617	2.634	0	0.219	0
	B	15.271	12.438	9.613	1.738	0	0	0
	C	15.621	12.51	9.703	2.323	0	0	0
19T	A	15.515	12.714	10.415	8.056	1.224	0	0
	B	15.45	12.643	9.558	2.706	0	0	0
	C	15.112	12.594	9.579	2.339	0	0	0
19U	A	15.445	12.792	10.203	8.869	6.663	4.981	3.504
	B	16.135	13.6685	10.805	9.388	1.298	0.278	0
	C	15.186	12.435	9.949	8.15	5.862	4.626	3.434
Control	A	15.262	11.592	9.575	7.826	5.959	4.291	3.329
	B	14.95	12.055	9.691	8.191	6.099	4.061	3.063
	C	15.258	12.128	9.88	8.34	6.292	4.131	3.171